Usefulness of Escherichia coli and Enterobacteriaceae as Process Hygiene Criteria in poultry - experimental study

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Usefulness of *Escherichia coli* and *Enterobacteriaceae* as Process Hygiene Criteria in poultry: experimental study[1]

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ABSTRACT

An experimental study in seven poultry slaughterhouses located in the EU was carried out with the objectives i) to collect relevant data on the variability of the counts of *E. coli* and *Enterobacteriaceae* on broiler carcasses sampled post evisceration and post chilling; ii) to collect information about the slaughterhouses visited and the sampled batches to explain the variability of the counts; iii) to compare *E. coli* and *Enterobacteriaceae* counts on the carcasses with their categorization in terms of levels of visual faecal contamination. The study quantified the level of *E. coli* and *Enterobacteriaceae* in 3 777 samples of neck skin, 1 887 obtained from carcasses at post evisceration and 1 890 at post chilling. In total, 97 out of the 3 777 broiler carcasses were classified as dirty in terms of levels of visual faecal contamination. The data collected were statistically analysed to assess the effect of slaughterhouse, batch and carcass variables on bacterial counts using a multilevel mixed linear model for hierarchical data. It was demonstrated that bacterial loads of both indicators were generally significantly lower at post chilling compared to post evisceration, and depended on the broiler's weight category. At post evisceration inspection, the inspector has the potential to visually classify carcasses as dirty; however, the probability of failure to recognise those carcasses with high bacterial counts is very high. The variables at batch and slaughterhouse level which affected the bacterial counts on carcasses were: weight category of the broilers (both the bacteria and sampling points), presence of discarded animals (both the bacteria at the post chilling), presence of intestinal ruptures (*E. coli* at post evisceration) and plucking method (both the bacteria at post evisceration).

KEY WORDS

Escherichia coli, *Enterobacteriaceae*, counts, poultry carcasses, slaughterhouse, risk factors

DISCLAIMER

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SUMMARY

A project entitled "Usefulness of *Escherichia coli* and *Enterobacteriaceae* as Process Hygiene Criteria in poultry" was awarded by EFSA to Istituto Zooprofilattico Sperimentale delle Venezie (Legnaro, Padova, Italy) with the purpose to collect available data on the indicator organisms *E. coli* or *Enterobacteriaceae* as Process Hygiene Indicators (PHI) for the main livestock species, based on a literature search and an experimental study, in this case in broiler slaughterhouses, located in the EU. The present document is the report on the experimental study. The report of the extensive literature review is published as two separate external scientific reports (Barco et al., 2014a; Barco et al., 2014b).

The tasks to be covered by the experimental study in poultry slaughterhouses located in the EU were to i) collect relevant data on the variability of the counts of *E. coli* and *Enterobacteriaceae* on neck and or breast skin of broiler carcasses sampled at the point of the chain where the presence of faecal contamination is assessed by the meat inspector as well as post chilling; ii) collect information, such as structural and managerial data about the slaughterhouses visited, as well as specific information about the sampled batches, to explain the variability of the counts; iii) compare *E. coli* and *Enterobacteriaceae* counts on the carcasses with and without visual faecal contamination.

In order to fulfil the objectives, the tenderer, with the collaboration of the National Food Institute, Technical University of Denmark, as subcontractor, carried out a sampling campaign in seven poultry slaughterhouses located in Denmark and Italy and considered to be illustrative of slaughterhouses that can typically be found in Europe.

The sampling plan, performed from April to the beginning of September 2013, allowed the quantification of the level of *E. coli* and *Enterobacteriaceae* in 3 777 samples of neck skin, 1 887 obtained from carcasses at post evisceration and 1 890 from carcasses at post chilling (sixty carcasses per batch were collected, except in one case). Out of the 3 777 broiler carcasses, 97 (86 belonging to the post evisceration group and 11 to the post chilling group) were classified as dirty in terms of levels of visual faecal contamination.

A total of 63 broiler batches (nine per slaughterhouse) were submitted to sampling, of which 33 were sampled at the beginning of the working day and 30 towards the end. All but two batches were classified as clean by veterinarians during the *ante mortem* evaluation.

Detailed data on slaughterhouses' management and procedures as well as detailed information on sampled batches were obtained in order to fulfil the second task.

The data collected were analysed using a multilevel mixed linear model for hierarchical data with the aim of investigating the effect of slaughterhouse, batch and carcass variables on *E. coli* and *Enterobacteriaceae* counts. In this context, slaughterhouse, batch and carcass identify three levels of clustered data sets. Such study design allows the investigation of whether covariates measured at each hierarchy level (level 1, carcass; level 2, batch; level 3, slaughterhouse) have an impact on the dependent variable (bacterial counts), which is measured at level 1 of the data structure.

Four models were designed with different aims. Briefly, models 3 (M3) and 4 (M4) are focused on explaining the variability of counts recorded at post chilling while model 2 (M2) is focused on explaining the variability of counts recorded at post evisceration. Model 1 (M1) is focused on providing explanation for possible differences between bacterial counts recorded both at post evisceration and post chilling.

The statistical analyses resulted in the following conclusions. *E. coli* and *Enterobacteriaceae* levels in broilers' neck skin are significantly lower at post chilling compared to post evisceration. The contamination level of both *E. coli* and *Enterobacteriaceae* recorded at both sampling points is lower in broilers belonging to the weight category 2-3 kg compared to the categories <2 kg and >3 kg, while no difference exists between the categories <2 kg and >3 kg.

The inspector has an extremely low probability of success in classifying a carcass with high bacterial counts as dirty simply by evaluating the visual faecal contamination level. Moreover, this ability is limited to the post evisceration stage. The presence of discarded animals at *post mortem* significantly affects bacterial counts recorded at post chilling. Specifically, batches without discarded animals show lower bacterial loads compared to batches with discarded animals. The presence of intestinal ruptures significantly affects only the *E. coli* loads observed at the post evisceration sampling point.

The type of plucking method significantly affects the contamination level: specifically *E. coli* and *Enterobacteriaceae* bacterial loads are lower when using the vertical or horizontal disk compared to the combined techniques (vertical, horizontal and counter-rotating).

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BACKGROUND AS PROVIDED BY EFSA

Regulation (EC) No 854/2004 of the European Parliament and of the Council lays down specific rules for the organization of official controls on products of animal origin intended for human consumption. Among others, inspection tasks within this Regulation include checks and analysis of food chain information, ante-mortem inspection and post-mortem inspection.

EFSA received a mandate from the Commission in May 2010 on the modernization of meat inspection, requesting a series of scientific opinions. The main scope of these opinions was to identify and rank the most relevant meat safety risks, to assess the strengths/weaknesses of the current meat inspection system, to propose alternative approaches for addressing current meat-safety risks, and to outline a generic framework for inspection, prevention and control (including related methodology) for the prioritized hazards that are not (sufficiently) covered by the current system.

Several species were to be considered. The scientific opinions on the public health hazards to be covered by inspection of swine meat (EFSA-Q-2010-00886) and poultry meat (EFSA-Q-2010-01469) were published in 2011 and 2012. Four more opinions concerning the inspection of meat from bovines/cattle (EFSA-Q-2011-00365), farmed game (EFSA-Q-2011-00366), small ruminants (EFSA-Q-2011-00365) and solipeds (EFSA-Q-2011-00367) were published in 2013.

In the scientific opinion on meat inspection of poultry, the BIOHAZ Panel concluded that *Campylobacter* spp. and *Salmonella* spp. are considered of high public health relevance for poultry meat inspection. Currently in the EU, the use of the food chain information for microbial food safety purposes is limited to *Salmonella* control, leading to *Salmonella*-positive flocks being slaughtered at the end of the day. In addition, samples of neck skin on broiler carcasses after chilling are used for the Process Hygiene Criteria laid down in Regulation No 2073/2005[3] as amended in Regulation 1086/2011[4].

Current post-mortem visual inspection is not able to detect any of the public health hazards identified as the main concerns for food safety. Visual detection of faecal contamination of carcasses at post-mortem inspection can be an indicator of slaughter hygiene. However, the high speed of the slaughter lines reduces the sensitivity of detection of carcass contamination by visual inspection and there is not a direct association with the occurrence of pathogens. Hence, other approaches to verify slaughter hygiene were considered as more appropriate by the BIOHAZ Panel.

The BIOHAZ Panel proposed recommending that the current visual inspection process is replaced by the establishment of targets for the main biological hazards on the carcass and by verification of the food business operators own hygiene management through the use of Process Hygiene Criteria (PHC). A potential approach for the latter is measuring *E. coli* or *Enterobacteriaceae* on poultry carcasses after chilling.

SPECIFIC OBJECTIVES AS PROVIDED BY EFSA

The purpose of the Service Contract is to provide EFSA with the available data on the indicator organisms *E. coli* or *Enterobacteriaceae* as Process Hygiene Indicators (PHI) for the main livestock species. Based on this literature search, an experimental study in broiler slaughterhouses located in the

[3] OJ L 338, 22.12.2005, p. 26.
[4] OJ L 281, 28.10.2011, p. 7.

EU should be designed and carried out to collect relevant data on these two indicator organisms. The ultimate aim is to support the purpose of potential PHC for evaluating process control in EU broiler slaughterhouses.

According to the Technical Specifications of the Service Contract CFT/EFSA/BIOHAZ/2012/03-CT1, the tasks to be covered are as follows:

- To carry out literature searches for data related to the main livestock species on (i) the presence of the indicator organisms *E. coli* and *Enterobacteriaceae* and their counts on carcasses during different stages in the slaughter processing line; (ii) information that could explain the variability of the counts of the indicator organisms and (iii) the potential relationship between the counts of indicator organisms and visual faecal contamination on carcasses;

- To perform an experimental study in broiler slaughterhouses located in the EU in order to (i) collect relevant data on the variability of the counts of *E. coli* and *Enterobacteriaceae* on broiler carcasses after chilling; (ii) collect information that could lead to interpretation of the variability of these counts and (iii) compare *E. coli* and *Enterobacteriaceae* counts on carcasses with and without visual faecal contamination.

The present document is the report on the experimental study in broiler slaughterhouses. The extensive literature search for available data on *E. coli* and *Enterobacteriaceae* on carcasses of poultry and pig and ruminant carcasses and are published as two separate external scientific reports (Barco et al., 2014a; Barco et al., 2014b).

This contract was awarded by EFSA to:

Contractor: Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Padova, Italy

Subcontractor: National Food Institute, Technical University of Denmark

Contract title: Usefulness of *Escherichia coli* and *Enterobacteriaceae* as Process Hygiene Criteria in poultry

Contract number: CFT/EFSA/BIOHAZ/2012/03

INTRODUCTION AND OBJECTIVES

In order to fulfil the experimental study objectives the tenderer, Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe), on the basis of the findings of literature search and of suggestions provided by EFSA, proposed a sampling design characterized by the followings main criteria:

- Selection of 7 poultry slaughterhouses: 2 located in Denmark and 5 in Italy, illustrative of European broiler slaughterhouses.

- Sampling of 540 broiler carcasses in each slaughterhouse for *E. coli* and *Enterobacteriaceae* quantification from neck skin samples.

- 9 batches, with two different levels of faecal contamination, to be sampled per slaughterhouse in different seasons (to investigate also a potential seasonality effect in counts variability) and at different times during the daily slaughtering activities (beginning and toward the end.)

- 60 carcasses, 30 after evisceration and 30 post chilling to be sampled per batch.

- All batches (at *ante mortem* and *post mortem* inspection) and each sampled carcass to be classified in terms of visual faecal contamination

- The sampling in the Danish slaughterhouses and the laboratory analysis of the collected broiler skin samples to be performed by the subcontractor, the National Food Institute, Technical University of Denmark.

The following activities have been carried out based on the approved sampling design:

-organization of a sampling campaign in selected slaughterhouses;

-development of two questionnaires to collect relevant data on the selected slaughterhouses and sampled broiler batches;

-preparation of a photo gallery to clarify criteria adopted within the study for classifying batches and carcasses;

-identification of a laboratory protocol suitable to determine levels of *E. coli* and *Enterobacteriaceae* on neck skin of broiler carcasses;

-development of a database for the storage and management of the collected data;

-identification of a statistical approach for describing results and identifying possible factors affecting bacterial counts;

As regards the skin samples to be collected for *E. coli* and *Enterobacteriaceae* quantification, the neck skin was considered to be suitable for the study, which had the added advantage that only the neck of the broiler carcasses was collected, and not the entire carcass, thus avoiding economic losses for the producers.

Before starting the sampling campaign, all supporting documents and organizational aspects were discussed with the subcontractor and EFSA.

1. MATERIALS AND METHODS

Details on all the phases of the experimental study are provided in the following paragraphs.

1.1 Organization of sampling in selected slaughterhouses

1.1.1 Slaughterhouses selection and recruitment

In total 7 slaughterhouses, of which 5 were in Italy and 2 in Denmark, were included in the study with the purpose of representing a range of throughputs, sizes, and ages that can typically be found in EU Member States.

Slaughterhouses were selected taking into account their potential capacity (size) and the available preliminary information on general technical characteristics. In particular, 3 large (>10,000,000 broilers slaughtered per year), 2 medium (between 1,000,000 and 4,999,999 broilers slaughtered per year) and 2 small (between 500,000 and 999,999 broilers slaughtered per year) sized European slaughterhouses were included in the study.

Meetings were organized, when needed, with the veterinarians responsible for the selected slaughterhouses, to explain the purposes of the study and the activities to be carried out in their premises and to provide all the useful information to guarantee the success of this part of the project. All the selected slaughterhouses welcomed the proposal.

1.1.2 Sampling management at slaughterhouse level

According to the approved sampling design, 9 broiler batches per slaughterhouse were sampled. For the purpose of this study, a batch of broilers was defined as a homogeneous group of broilers raised on one farm and transported to the slaughterhouse on one truck.

The sampling strategy at batch level was as follows: batches had to be sampled in 9 separated visits (where visit corresponds to a sampling day) or alternatively during the same visit/day a maximum of two batches could be sampled.

In order to be able to investigate the seasonality effect in *E. coli* and *Enterobacteriaceae* counts, the visits were carried out both in the spring and in the summer period (according to the project constraints). Considering the potential climatic differences between Italy and Denmark, in order to have an objective classification, temperature on the day of sampling was recorded.

To obtain data to evaluate the slaughterhouse effect on the variability of *E. coli* and *Enterobacteriaceae* counts, the sampling agenda was organized in order to collect, in the same slaughterhouse, samples at different slaughtering times, and in particular, half of the batches were sampled at the beginning of the slaughtering day and half at the end.

1.1.3 Sampling management at batch level

The sampling of each broiler batch had to be carried out as follows: 60 carcasses (or broiler necks) per batch, of which 30 were from immediately after evisceration and 30 from immediately after chilling. Chilling, for this study, is considered the phase of the abatement of the temperature before moving the carcasses into the cold rooms for stocking. Carcasses within each batch were collected applying systematic random sampling based on a regular time interval according to the slaughter line speed, in order to guarantee the representativeness of the entire batch.

The sampling had to be organized to sample batches which had been differently classified at the *ante mortem* inspection, in terms of visual faecal contamination. Batches were classified as clean or as contaminated, and both had to be sampled while still respecting the other criteria described above. In order to use standardized criteria for the *ante mortem* visual classification, reference images were provided (photo gallery is included in Appendix D).

1.1.4 Sampling management at carcass level

In order to avoid any cross-contamination among the 60 carcasses sampled each time, plastic gloves and equipment used to collect the samples (knifes or scissors, plastic bags) were replaced or cleaned

and disinfected between each carcass. The sample for *E. coli* and *Enterobacteriaceae* quantification corresponded to 10 grams of neck skin.

Each sampled carcass was classified in terms of visual faecal contamination both according to the criteria currently used by the meat inspectors and experimental study criteria based on two levels: "clean" and "contaminated". Standardized study criteria for the carcasses visual classification was guaranteed by provision of reference images (photo gallery is provided in the Appendix E).

The temperature, external in case of the post evisceration sampling point and both internal and external in case of the post chilling sampling point, was recorded for one carcass per batch.

To avoid biases due to lack of experience, only trained personnel were enrolled to collect samples and relevant data.

1.2 Collection of relevant data

Two questionnaires were designed to collect relevant data at slaughterhouse and batch level.

Relevant information to be included in the questionnaires was selected according to information gathered through the extensive literature review (Barco et al., 2014b) and incorporating the suggestions of veterinarians responsible for the selected slaughterhouses that were contacted and involved at the very beginning of the study. The veterinarians were requested to complete the slaughterhouse questionnaire at the first visit, while the batch questionnaire was filled in during each visit.

The slaughterhouse questionnaire included items related to: slaughterhouse capacity, type of birds slaughtered, features of the slaughter line/s including stunning, killing, scalding, plucking, evisceration, decontamination washing and chilling methods and use of information on batch positivity to foodborne pathogens for planning the order in which batches are slaughtered. This questionnaire is provided in Appendix A.

The batch questionnaire included items related to: origin of the flock, weather conditions during transport, time of catching and loading, temperature on the day of sampling, features of the batch including number, age and weight of the birds, sanitary *status* toward foodborne pathogens and feed withdrawal duration, additionally information on the slaughter process is required such as slaughter duration, number of discarded animals and prevalent reasons of discard and percentage of intestinal ruptures. This questionnaire is provided in Appendix B.

The information regarding the cleanness *status* of sampled carcasses had to be reported in a separate form (Appendix C).

1.3 Identification of a laboratory protocol

The following laboratory protocol was used for the purposes of the study. Neck skin obtained from each neck or carcass sample was kept refrigerated during transport and at the laboratory until the beginning of the analyses. Skin samples were analysed as soon as possible and in all cases within 24 hours of the sampling.

E. coli and *Enterobacteriaceae* counts were determined using the Petrifilm method which is validated against the ISO (*E. coli*) 16649-2:2001 and (*Enterobacteriaceae*) ISO 21528-2:2004, and which was one of the preferential methods for indicator bacteria quantification according to the literature review.

The final number of *E. coli* and *Enterobacteriaceae* was derived using the formula reported in the ISO 7218:2007:

$$C = \frac{N_1 + N_2}{V \cdot d_1 \cdot 1.1}$$

where

N_1 and N_2= number of CFU counted in plates 1 and 2

V= volume deposited in both plates (ml)

d_1= dilution rate of plate 1 (meaning the dilution of the plate referred to N_1)

The formula requires the use of the first two countable plates, preferably in the range 10-150, as recommended by ISO 16649-2:2001 (*E. coli*) and ISO 21528-2:2004 (*Enterobacteriaceae*). Results are expressed as CFU/g.

1.4 Development of a database for the management of the collected data

A dedicated database through a web interface was built to store and manage data arising from the questionnaires and bacterial quantification. Firstly, a conceptual data model, that reflected the structure of the information gathered via the questionnaires, was produced and then, based on this, a relational database model using SQL language was implemented.

Data model was translated into a XML schema in order to allow data exchange with EFSA.

A database management system (DBMS), providing a web interface between the users and the database, was developed in PHP. The DBMS allows insertion and modification of data, retrieval of data in customized reports, and export of data in the defined XML format.

Access control to the database was managed by assigning individual and group privileges, in order to prevent unauthorized users from viewing or updating the data.

Data security was guaranteed by access logging with user name and password.

1.5 Statistical methodology

Statistical analysis was performed with the following objectives:

a) to describe the distribution of the counts of *E. coli* and *Enterobacteriaceae* on neck skin of broiler carcasses sampled at post evisceration and post chilling, focusing on the comparison of bacterial loads on carcasses according to their categorisation in terms of levels of visual faecal contamination;

b) to describe the data about the slaughterhouses visited, as well as specific information about the sampled batches; and

c) to identify factors at slaughterhouse, batch and carcass level that significantly affect bacterial loads.

The first two aims were covered by the descriptive statistical analysis while the third one was covered by statistical models.

Quantitative data, colony forming units (cfu) of *E. coli* and *Enterobacteriaceae* per g of neck skin were $\log_{10}$-transformed before statistical analysis.

1.5.1 Descriptive statistical analysis

Descriptive statistical analysis was performed to summarise data on slaughterhouses, batches and carcass characteristics. Data on slaughterhouse and batch characteristics were summarised in tables and graphs.

As regards outputs of *E. coli* and *Enterobacteriaceae* counts ($\log_{10}$ cfu/g), box-plots were used to synthesize the data, providing the principal measures of central tendency and dispersion. Specifically the diagram comprises a box with horizontal limits defining the upper and lower quartiles representing the interquartile range (thus enclosing the central 50% of the observations), with the median marked by a horizontal line within the box. The whiskers are vertical lines extending from the box as low as the 2.5^{th} percentile and as high as the 97.5^{th} percentile. Extreme values are indicated by dots and mean values, when reported, are indicated by the symbol "*".

A spline function was developed to investigate the potential relationship between the bacterial counts observed on carcasses at the post chilling sampling point and the average bacterial counts at batch level observed at the post evisceration sampling point for both *E. coli* and *Enterobacteriaceae*.

The quantile-quantile plots (Q-Q plots), that compare the ordered counts of *E. coli* and *Enterobacteriaceae* with quantiles of a specific theoretical distribution were used to verify the assumption of data normality, which is required by the selected statistical model.

1.5.2 Statistical model

The objective of the statistical analysis was to relate the variability of the *E. coli* and *Enterobacteriaceae* counts ($\log_{10}$ cfu/g) (dependent variable) to the effects of covariates measured at each level of the dataset (slaughterhouse, batch and carcass level).

The statistical model used to investigate the effect of slaughterhouse, batch and carcass variables on *E. coli* and *Enterobacteriaceae* counts is a multilevel mixed linear model for hierarchical data (West et al., 2007). In this context, slaughterhouse, batch and carcass identify a three-level clustered data set. The units of analysis, i.e. the carcasses (Level 1) are nested within randomly sampled batches (Level 2), which are in turn nested within randomly sampled slaughterhouses (Level 3).

Such study design allows the investigation of whether covariates measured at each hierarchy level have an impact on the dependent variable, which is measured at Level 1 of the data structure.

The covariates for each hierarchical level derive from the information obtained through the slaughterhouse and batch questionnaires and carcass form.

The general specification of the model:

$$\log(counts)_{ijk} = \underline{x}'_{ijk}\underline{\beta} + \upsilon_{j/k} + u_k + \varepsilon_{ijk}$$

where

$\log(counts)_{ijk}$ represents the value of dependent variable for the carcass *i* in batch *j* within slaughterhouse *k*.

x_{ijk} is the vector of the covariates for the *i*-th carcass in batch *j* within slaughterhouse *k*.

β represents the vector of fixed intercept and fixed effects of the covariates at different levels

u_k is the random effect associated with the intercept for slaughterhouse *k*

$u_{j|k}$ is the random effect associated with the intercept for batch *j* within slaughterhouse *k*; and

ε_{ijk} represents the residual.

The distribution of the random effects associated with the slaughterhouse is:

$$u_k \sim \mathrm{N}(0, \sigma^2_{\mathrm{int:slaugh}})$$

where $\sigma^2_{\mathrm{int:slaugh}}$ represents the variance of the slaughterhouse-specific random intercepts.

The distribution of the random effects associated with batches nested within a given slaughterhouse is described as:

$$\upsilon_{j/k} \sim \mathrm{N}(0, \sigma^2_{\mathrm{int:batch}})$$

where $\sigma^2_{\mathrm{int:batch}}$ represents the variance of the random batch-specific intercepts at any given slaughterhouse. This between-batch variance is assumed to be constant for all slaughterhouses.

The distribution of the residuals associated with the carcass-level observations is given by:

$$\varepsilon_{ijk} \sim \mathrm{N}(0, \sigma^2)$$

where σ^2 represents the residual variance.

The random effects, u_k, associated with slaughterhouse, the random effects, $u_{j|k}$, associated with batch nested within slaughterhouse, and the residuals, ε_{ijk} are assumed to be all mutually independent.

Four models (identified as M1, M2, M3, M4) were designed with different aims and the variables included in each model were selected according to the model purpose. Model 1 (M1) focused on explaining possible differences between bacterial counts recorded both at post evisceration and post chilling; model 2 (M2) focused on explaining the variability of counts recorded at post evisceration while models 3 (M3) and 4 (M4) focused on explaining the variability of counts recorded at post chilling.

Details on the variables included in each model are provided below.

M1: The dependent variable consisted of all the observed $\log_{10}$ counts. A binary variable to differentiate counts recorded on carcasses sampled at the **post chilling and post evisceration sampling points** was introduced at level 1. In this model, all the variables at batch level were taken into account, whereas for the slaughterhouse level, variables related to slaughter phases following after the evisceration step were excluded, since they potentially affect only counts recorded at the post chilling sampling point.

M2: The dependent variable consisted of all the $\log_{10}$ counts observed on carcasses sampled at the **post evisceration sampling point**. In this model, all the variables at batch level were taken into account, whereas, for the slaughterhouse level, variables related to slaughter phases following after the evisceration step were excluded, since they did not affect the bacterial loads at the post evisceration sampling point at all.

M3: The dependent variable was the $\log_{10}$ counts observed on carcasses sampled at the **post chilling sampling point**. In this model, all the variables at batch and slaughterhouse levels were taken into account.

M4: The dependent variable was the $\log_{10}$ counts observed on carcasses sampled at the **post chilling sampling point**. In this model, all the variables at the batch and slaughterhouse level were taken into account. Additionally, a new variable, corresponding to the average $\log_{10}$ counts on carcasses sampled at the post evisceration sampling point, was created at batch level in order to evaluate any possible relationship between post chilling and post evisceration bacterial counts.

The data were analysed according to the following subsequential steps:

Step 1: *Fit a three-level model with a fixed intercept, and random effects associated with the intercept for batches (Level 2) and slaughterhouses (Level 3) to obtain the model A (MA), and decide whether to keep the random intercepts for batches*

To obtain the estimate of the initial variance components, i.e., the variance of the random effects at the slaughterhouse level and the batch level, and the residual variance at the carcass level, a model that was not conditioned by any fixed effects other than the intercept was considered. This model includes a fixed overall intercept, random effects associated with the intercept for batches within slaughterhouses and random effects associated with the intercept for slaughterhouses. The variance component estimates from the model allow estimation of the intra-class correlation coefficients (ICCs) of $\log_{10}$ counts responses at the slaughterhouse level and at the batch level to describe the similarity (homogeneity) of observed responses within the considered clusters. The slaughterhouse-level ICC is defined as the proportion of the total random variation in the observed responses due to the variance of the random slaughterhouse effects.

$$\mathrm{ICC}_{\mathrm{slaugh}} = \frac{\sigma^2_{\mathrm{int:slaugh}}}{\sigma^2_{\mathrm{int:slaugh}} + \sigma^2_{\mathrm{int:batch}} + \sigma^2}$$

Similarly, the batch-level ICC is defined as the proportion of the total random variation due to random between-slaughterhouse and between-batch variation:

$$\mathrm{ICC}_{\mathrm{batch}} = \frac{\sigma^2_{\mathrm{int:slaugh}} + \sigma^2_{\mathrm{int:batch}}}{\sigma^2_{\mathrm{int:slaugh}} + \sigma^2_{\mathrm{int:batch}} + \sigma^2}$$

Furthermore, the restricted maximum likelihood (REML) ratio between this last model and the model without the random batch effects facilitates the decision about whether to keep the hierarchical structure of the data. The null and the alternative hypotheses to test were:

$$H_0 : \sigma^2_{\mathrm{int:batch}} = 0$$
$$H_1 : \sigma^2_{\mathrm{int:batch}} > 0$$

The test statistic in this case has an asymptotic null distribution that is a mixture of χ^2_0 and χ^2_1 distributions, each having an equal weight of 0.5.

Step 2. *Add fixed effects associated with covariates measured on the carcasses to the MA, to obtain model B (MB), evaluate the reduction in the residual variance and decide whether to retain the effects of the Level 1 covariates in the model.*

Step 3: *Add fixed effects associated with the covariates measured at Level 2 (batch) to create model C (MC), evaluate the reduction in the residual variance and decide whether to retain the effects of the Level 2 covariates in the model.*

Step 4: *Add fixed effects associated with the covariate measured at Level 3 (slaughterhouse) to MC to create model D (MD), evaluate the reduction in the residual variance and decide whether to retain the effects of the Level 3 covariates in the model.*

Step 5: *Add fixed cross-level interactions effect associated with the covariate measured at different levels to MD to create model E (ME), evaluate the reduction in the residual variance and decide whether to retain the cross-level interaction in the model.*

The forward model selection procedure was applied to identify the variables to be included in the proposed models. The interactions among different variables were evaluated according to their potential biological relevance, which was assessed through expert consultations. To evaluate the significance of the overall effect of fixed factors specified in the model, Type III F-tests, in which the significance of each term is tested conditionally on the fixed effects of all the other terms in the model, were applied from steps 2 to 5. P-values smaller than 0.10 were considered to be significant. To calculate the denominator degrees of freedom (df) for F-tests in Type III tests of fixed effects, the Satterthwaite approximation was used, since it guarantees a more accurate F-test approximation, and hence a more accurate p-value for the F-test. For each fixed factor of the mixed models, post-hoc pair-wise comparisons of the least-squares means were performed to further clarify those differences. In the case of multiple tests, the adjusted p-values for the FDR method (False Discovery Rate) are provided (Benjamini and Hochberg, 1995).

Step 6: *Analysis of Studentized conditional and Studentized marginal residuals to evaluate the goodness of final model*

The estimated fixed effects based on the restricted maximum likelihood (REML) estimation were used to calculate the predicted values of $\log_{10}$ counts. Two different sets of predicted values were considered: conditional predicted values based on the estimated fixed effects and on the Empirical Best Linear Unbiased Predictor (EBLUPs) of the random slaughterhouse and batch effects, and marginal predicted values based only on the estimated fixed effects. The difference between observed and conditional predicted values or marginal predicted values generates the conditional (r_{ijk}^{C}) and marginal raw residuals (r_{ijk}^{M}) respectively. The conditional Studentized residuals are defined as:

$$r_{ijk}^{C,St} = \frac{r_{ijk}^{C}}{\sqrt{\widehat{Var}[r_{ijk}^{C}]}}$$

where

$$r_{ijk}^{C} = \log(counts)_{ijk} - \underline{x}'_{ijk}\,\underline{\widehat{\beta}} + \widehat{\upsilon}_{j/k} + \widehat{u}_{k}$$

The marginal Studentized residuals, defined as

$$r_{ijk}^{M,St} = \frac{r_{ijk}^{M}}{\sqrt{\widehat{Var}[r_{ijk}^{C}]}}$$

where

$$r_{ijk}^{M} = \log(counts)_{ijk} - \underline{x}'_{ijk}\,\underline{\widehat{\beta}}$$

This type of residuals was preferred in this analysis since it is considered more appropriate than the raw residuals in examining model assumptions and detecting outliers and potentially influential points.

The conditional Studentized residuals were used to assess the assumptions of normality and constant variance. Marginal Studentized residuals were used to check fixed effects, as is usual in linear regression models.

QQ-plot, residuals distribution and scatterplot of the residuals vs. the fitted were plotted to verify the assumption of normality and homoschedasticity.

To compare non-nested multilevel mixed models, the Bayesian information criterion **(**BIC) was used.

Only the models which satisfied the parameter convergence, log-likelihood convergence and Hessian convergence were kept (West et al., 2007).

PROC MIXED of SAS 9.3 software was used to fit the models (Littell et al., 2006).

2. RESULTS

2.1 Sampling

The sampling was carried out between April and September 2013.

Nine batches were sampled in each of the seven selected slaughterhouses. Out of the 63 batches examined, 33 were sampled at the beginning of the working day and 30 towards the end of the working day. Altogether, 61 batches were classified as clean by veterinarians during *ante mortem* evaluation; in contrast, only two batches were classified as dirty at the *ante mortem* inspection. The judgment of the veterinarians in charge of sample collection concerning the cleanliness of batches was always carried out in accordance with the criteria adopted routinely in the slaughterhouse.

Sixty carcasses per batch were sampled during each slaughterhouse visit, except in one case, at slaughterhouse 3, where during the sampling of batch no. 6, three carcasses were missed at the post evisceration sampling point.

A total of 3 777 samples of neck skin were submitted for laboratory analysis, 1 887 obtained from carcasses at post evisceration and 1 890 obtained from carcasses at post chilling. Despite the effort to collect dirty carcasses without affecting the randomization criteria, only 97 out of the 3 777 sampled carcasses were classified as dirty, 86 belonging to the post evisceration group and 11 to the post chilling group. There was complete agreement between the samplers and the veterinarians responsible in the slaughterhouses over classifying the carcasses.

In Figure 1, the number of dirty carcasses per slaughterhouse is shown.

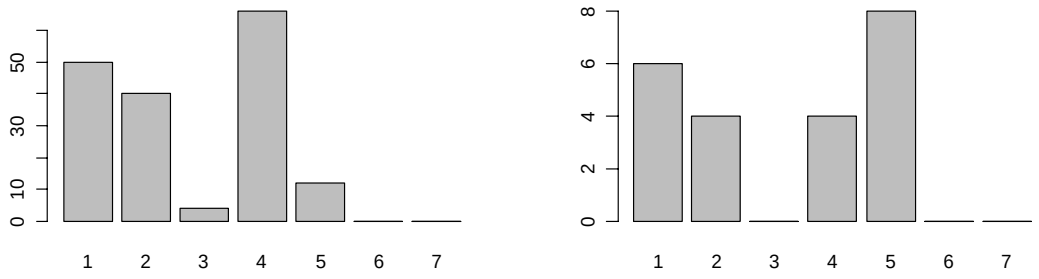


Figure 1: Number of dirty carcasses per slaughterhouse at post evisceration (left) and at post chilling (right)

2.2 Descriptive statistical analysis

Slaughterhouses, batches and carcass characteristics

Slaughterhouses, batches and carcasses were identified with numerical and alpha numerical codes according to the following: slaughterhouses from 1 to 7 (identified in the Tables as IDs); batches within each slaughterhouse from 1 to 9 and carcasses within each batch from 1PO-EV (whereas PO stands for post and EV stands for evisceration) to 30 PO-EV as regards carcasses sampled after

evisceration and from 1PO-CH (whereas PO stands for post and CH stands for chilling) to 30 PO-CH as regards carcasses sampled after chilling.

In three samples collected at the post chilling sampling point, *E. coli* counts were less than 10 cfu/g; for statistical analysis these data were considered equal to 10 cfu/g.

2.2.1 Slaughterhouse information

As regards the size of the seven selected slaughterhouses, three of them (2, 6 and 7) slaughtered more than 10,000,000 broilers per year, two (4 and 5) from 1,000,000 to 4,999,999 broilers and two (1 and 3) less than 999,999 broilers. Therefore, three slaughterhouses may be considered large in size, two medium and two small (Table 1).

Three slaughterhouses slaughtered birds other than poultry; in particular the smaller slaughterhouses also slaughtered layers, guinea fowls, ducks, geese and game birds (Table 1).

Table 1: Yearly number of slaughtered animals by species

IDs	Broilers	Other species than broilers					
		Layers	Breeders	Guinea fowl	Ducks	Geese	Game birds
1	500,000-999,999	-	-	< 100,000	-	-	-
2	> 10,000,000	-	-	-	-	-	-
3	< 100,000	< 100,000	-	< 100,000	< 100,000	< 100,000	< 100,000
4	1,000,000-4,999,999	1,000,000-4,999,999	< 100,000	< 100,000	-	-	-
5	1,000,000-4,999,999	-	-	-	-	-	-
6	> 10,000,000	-	-	-	-	-	-
7	> 10,000,000	-	-	-	-	-	-

The daily number of broilers slaughtered on average ranged from 140,000 to 180,000 for slaughterhouses 2, 6 an 7, operating five or even six days per week; from 8,000 to 18,000 for slaughterhouses 4 and 5, operating from three to six days per week and from 300 to 5,000 for the slaughterhouses 1 and 3, which slaughtered three and two days per week, respectively.

The smaller slaughterhouses, 1 and 3, slaughtered three batches and one batch per day on average, respectively; the medium slaughterhouses, 4 and 5, five and two batches, respectively, and the larger slaughterhouses 2, 6, 7 slaughtered eight, seven and four batches on average per day, respectively.

The working hours per day (on average) and the number of operators depended on the slaughterhouse size and ranged from 4 to 16 and from 5 to 93, respectively. Daily working shifts are organized in all the selected slaughterhouses according to the length of the working day and the number of operators (a minimum of 4 hours and a maximum of 8 hours were declared).

The line speed was faster in larger slaughterhouses, which were able to slaughter up to 205 broilers per minute. Additionally, the weight of the broilers did not affect the line speed when different weight categories were slaughtered, except for slaughterhouse 4 where the line speed was shorter for the weight category >3 kg (55 broilers/ minute versus 75/broilers minute).

In Table 2, details on scalding, plucking and evisceration methods are provided. It is important to underline that the stunning method adopted by all the selected slaughterhouses was electronarcosis in water, with the exception of slaughterhouse 3.

Table 2: Technical information about the slaughterhouses

IDs	Stunning method	Scalding method: Bath type	Scalding T° (°C)	scalding phase (minutes)			Plucking method	Plucking completed by hands	Evisceration method
				<2 kg	2-3 kg	>3 kg			
1	Electric water bath	Single-bath without counterflow	50.6		1	1	Vertical disk and Counter-rotating disk	Yes	Automatic drawing completed by hands
2	Electric water bath	Multi-bath counterflow	[53; 53; 53] *	2			Vertical disk and Counter-rotating disk and horizontal disk	Yes	Automatic drawing
3	Electric	Single-bath counterflow	50		2		Vertical disk	Yes	Automatic through suction pump
4	Electric water bath	Single-bath without counterflow	48.5	4	4	5	Vertical disk and counter-rotating disk and horizontal disk	No	Automatic drawing completed by hands
5	Electric water bath	Single-bath without counterflow	52	3		4	Horizontal disk	Yes	Automatic drawing completed by hands
6	Electric water bath	Multi-bath counterflow	[52; 54; 53] *	2	2		Vertical disk	No	Automatic drawing
7	Electric water bath	Multi-bath without counterflow	[58; 57; 56] *		2		Vertical disk	No	Automatic drawing

* In these cases the water temperature for each bath is indicated

The killing method consisted of cutting both carotids for all the slaughterhouses (slaughterhouse 2 did not provide details on this).

All slaughterhouses declared that they have one slaughter line in more than one room with the exception of slaughterhouse 3 (two lines) and slaughterhouse 7 (just one room). As regards the operation of washing carcasses between plucking and evisceration, only slaughterhouse 4 declared that it undertakes this procedure. Slaughterhouse 7 did not provide details on this. Slaughterhouses 1, 2, 4 and 7 declared that washing the carcasses after evisceration is undertaken, but, in the case of the slaughterhouse 7 only, this procedure took place between the evisceration and the inspection point.

The inspection point was always located where evisceration takes place, with the exception of slaughterhouse 3, where it was located following the defeathering phase; in particular in slaughterhouses 1, 2, 4, 5 and 7, the inspection point was located immediately after the evisceration phase, while in slaughterhouse 6 it was immediately before.

Washing the carcasses for decontamination purposes was standard procedure only in slaughterhouses 1 and 2; the former used water at a temperature of 15 °C and applied an external shower, the latter used water at 20 °C and applied high pressure for internal and intra-cavity washing.

In Table 3, details are provided as regards the chilling phase. In all slaughterhouses, cold air was used to lower the carcass temperature, and tunnel cooling is the method used by six out of the seven selected slaughterhouses; the chilling temperature ranged from -5 °C to 4 °C.

As regards the frequency of line/s clean up, slaughterhouses 1, 4, 6 and 7 declared that they undertake cleaning between slaughtering shifts, slaughterhouse 5 cleaned between breaks, slaughterhouse 3 cleaned at changing of species /category and slaughterhouse 2 cleaned between operators' shifts, between breaks and at changing of species/category.

All the slaughterhouses declared that the plant itself and equipment are all (or most of them) maintained in good order.

Finally all the slaughterhouses stated that in the month prior to the study, the event of observing intestinal leakage had happened "sometime".

The slaughterhouse questionnaire included a question on the policy of planning slaughter on the basis of the health *status* of the flock, in particular focused on flock positivity to foodborne pathogens. Four of the slaughterhouses (1, 2, 3, 4), declared that they plan the order of slaughter according to the health *status* of the batches, specifically whether the flock has been identified as *Salmonella* positive; in addition, slaughterhouses 2 and 4 base batch slaughter order on flock positivity for *Salmonella* Enteritidis and Typhimurium.

Table 3: Chilling phase technical information

IDs	Chilling method	Chilling technique	Chilling phase (minutes)			Chilling temperature (°C)	Chilling completed in refrigeration room
			<2 kg	2-3 kg	>3 kg		
1	Air	tunnel	65	65	65	0.6	Yes
2	Air	tunnel	180			[4; 0.5; 1]*	No
3	Air	refrigerating room		30		4	Yes
4	Air	tunnel	160	160	160	[0; -5; -3]*	Yes
5	Air	tunnel	55		55	0	Yes
6	Air	tunnel	150	150		2	Yes
7	Air	tunnel		180		[0; 0.2; 0.2]*	Yes

* In these cases the chilling temperature is not homogeneous, thus the temperatures in the starting, central and final parts of the tunnel are indicated

2.2.2 Batch information

Nine batches were sampled in each of the seven selected slaughterhouses.

The sampling campaign started in the second week of April and finished the second week of September; contract constraints were taken into account. The number of batches sampled per month is reported in Figure 2. On average, 1.5 batches were sampled per month per slaughterhouse.

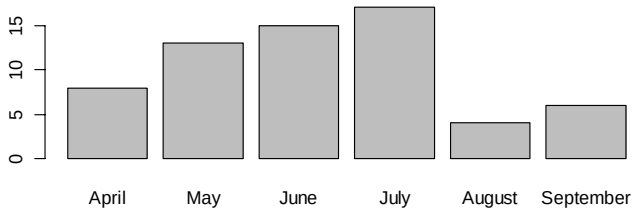


Figure 2: Monthly number of batches sampled in each slaughterhouse

The temperatures registered at sampling day ranged from 7 to 27 °C (mean temperature 19.1 °C). The temperatures registered in the slaughterhouses lairage pens where broilers were kept ranged from 13 to 28 °C (mean temperature 19.3 °C). As regards the weather conditions during broilers' transport from the farms to the slaughterhouse, which could affect the animals' welfare and the cleanness *status*, in most of the cases (36), it was sunny or clear, in 16 cases cloudy, in 1 case foggy, in 8 cases it was lightly raining and in only 2 cases overcast.

The time dedicated to catch and load the birds ranged from 59 minutes to 18.8 hours (mean equal to 366 minutes) and from 20 minutes to 18.8 hours (mean equal to 207 minutes) respectively. The information on the catching time was provided only for 18 batches. The duration of slaughter ranged from 25 minutes to 8.75 hours. Further details are provided in Table 7 of Appendix F.

Out of the 63 batches collected, 33 were sampled at the beginning of the working day and 30 towards the end of the working day (details are provided in Table 1 of Appendix F).

Altogether, 61 batches were classified as clean by veterinarians during *ante mortem* inspection; in contrast only two batches were classified as dirty at *ante mortem* inspection. The judgments of the veterinarians in charge of sample collection, concerning the cleanliness of batches, were always in accordance with the criteria adopted routinely in each slaughterhouse. All of the batches, which were classified as clean at *post mortem* by the sampler veterinarians, were also classified as clean by the official veterinarians.

In Figures 3 and 4, the distribution of *E. coli* and *Enterobacteriaceae* counts is summarized according to the batch classification based on the visual faecal contamination at *ante mortem* inspection.

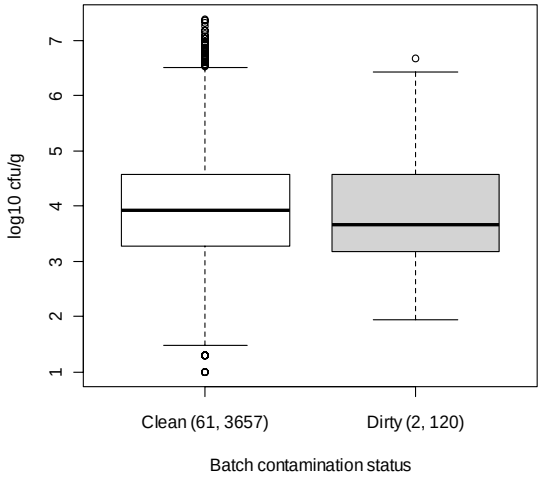


Figure 3: *E. coli* counts on broiler carcasses (log_{10} cfu/g) categorised by the batch contamination *status*. The number of batches and the corresponding number of sampled carcasses are reported in brackets.

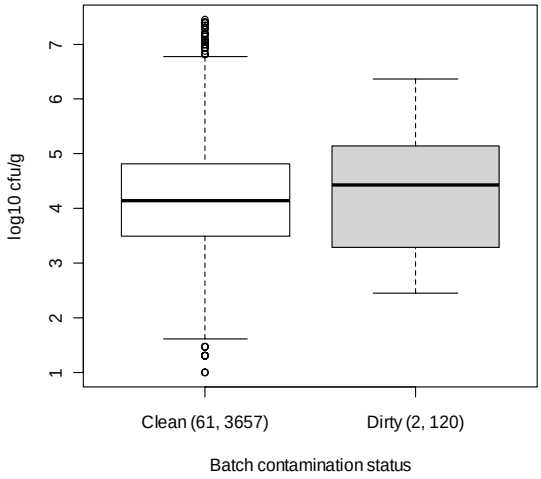


Figure 4: *Enterobacteriaceae* counts on broiler carcasses (log_{10} cfu/g) categorised by the batch contamination *status*. The number of batches and the corresponding number of sampled carcasses are reported in brackets.

Since only 2 batches out of 63 were classified as dirty, direct comparison of the count distribution between the two groups is not meaningful; thus, an appropriate statistical test has been applied to the dataset, in order to clarify the potential differences of indicator bacteria loads in carcasses belonging to these two types of batches (details are reported in Appendix G).

Data were obtained on the type of production adopted by the holdings of origin of the sampled batches: only two out of the 63 sampled batches consisted of broilers raised on organic farms, one batch of which was delivered to slaughterhouse 1 and the other one to slaughterhouse 6.

In almost all cases (53/63), the batches submitted to sampling represented just part of an entire flock. Altogether, 23 out of the 63 batches consisted of birds belonging to the weight category 2-3 kg, 21 to the weight category > 3 kg and 19 to the category <2 kg. Further details are provided in Table 2 of Appendix F. In the majority of the cases, batches were considered to be homogeneous as regards the weight of the broilers, while only 4 batches (one delivered to slaughterhouse 2, one to slaughterhouse 4 and two to slaughterhouse 6) were considered not to be homogeneous.

The number of animals in the batches submitted to sampling ranged from 330 to 41,100 (median number 5,120 and mean number 10,430). The age of the animals ranged from 32 to 120 days (median age 40 and mean age 50.7). Further details as regards the number of animals per batch and the age of the broilers are reported in Tables 3 and 4 of Appendix F, respectively.

The number of dead animals on arrival ranged from 0 to 184 (median number 6 and mean number 24.3) (details for each slaughterhouse are provided in Table 5 of Appendix F).

To have an objective criterion related to the welfare *status* of the animals before being slaughtered, the number of broilers per crate in terms of square metre was recorded: this indicator ranged between 12 to 36 (mean number of broilers per square metre was 20.9). Additional details are available in Table 6 of Appendix F.

In the batch questionnaire, information on the feed withdrawal duration both in terms of time, in case of availability of this data, and in terms of presence of feed in the crop observed at the slaughterhouse was provided: the number of hours of feed withdrawal was provided only for 20 batches, whereas for all 63 batches, the presence of feed in the crop was recorded; feed was present in the crops of less than 10 % of the birds.

The number of discarded animals per batch was recorded, and the percentage ranged from 0 % to 0.02 % (median percentage 0.003 and mean percentage 0.004). Only in slaughterhouse 3 no discarded animals were recorded. As regards the reasons for carcass rejection, the most frequent cause was cachexia (25 cases), followed by muscle alterations (7 cases), hepatitis (4 cases), abscesses and ascites (2 and 1 case respectively); thus, all these causes were related to problems during farming. Poor bleeding and traumatic lesions, which occurred during slaughter, were reported as causes for rejection of two carcasses.

Intestinal ruptures were present in 30 out of the 62 batches (for one batch this information was not reported) with a frequency from 0.003 to 2.5 %; only in one batch 10% of the broilers were rejected due to intestinal ruptures.

Considering the presence of intestinal ruptures according to the weight category of the batches, 14 were in the category 2-3 kg, 6 in the category > 3 kg and 10 in the category < 2 kg.

All the batches were declared to be negative as regards *Salmonella* spp., while as regards the sanitary *status* towards *Campylobacter spp.,* in twelve cases, the batches were declared to be positive and in 51 cases no information was reported.

2.2.3 Carcass information

Sixty carcasses per batch were sampled during each visit except in one case, at slaughterhouse 3, where, during the sampling of batch no. 6, three carcasses were missed at the post evisceration point of sampling.

One carcass in each batch was selected to evaluate the external temperature post evisceration and another was selected to evaluate the temperature, both internal and external post chilling. At the post evisceration sampling point, temperatures ranged from 26 to 42 °C (mean temperature 34.4 °C); at post chilling the external temperature was between 0 and 29 °C (mean temperature 7.5 °C) and the internal between 0 and 27 °C (mean temperature 10.2 °C) (further details are described in Table 1 of Appendix H) .

A total of 3 777 samples of neck skin (1 887 obtained from carcasses at post evisceration and 1 890 obtained from carcasses at post chilling) were submitted for laboratory analysis to quantify *E. coli* and *Enterobacteriaceae*. Despite the effort to collect dirty carcasses without affecting randomization criteria, only 97 out of the 3 777 sampled carcasses were classified as dirty, 86 belonging to the post evisceration group and 11 to the post chilling group (Figure 1).

Out of the 97 dirty carcasses, 11 were detected in batches with broilers belonging to the 2-3 kg weight category, 26 in batches with broilers < 2 kg and 60 in the category > 3 kg.

Data on the levels ($\log_{10}$ cfu/g) of *E. coli* and *Enterobacteriaceae* in the sampled carcasses are summarised in box-plots below.

Bacterial loads observed in the seven slaughterhouses are reported in Tables 2 and 3 of Appendix H for *E. coli* and *Enterobacteriaceae* respectively. As regards carcasses sampled at the post evisceration sampling point, *E. coli* loads ($\log_{10}$ cfu/g) ranged from 1.30 to 7.38 in clean carcasses and from 2.40 to 7.04 in dirty carcasses, respectively. For carcasses sampled at the post chilling sampling point, *E. coli* loads ($\log_{10}$ cfu/g) ranged from 1 to 6.95 in clean carcasses and from 2.65 to 5.28 in dirty carcasses, respectively. As regards carcasses sampled at the post evisceration sampling point, *Enterobacteriaceae* loads ($\log_{10}$ cfu/g) ranged from 1.48 to 7.45 in clean carcasses and from 2.45 to 7.26 in dirty carcasses, respectively. For carcasses sampled at the post chilling sampling point, *Enterobacteriaceae* loads ($\log_{10}$ cfu/g) ranged from 1 to 7.08 in clean carcasses and from 3.54 to 5.18 in dirty carcasses, respectively.

Observing the data of the two indicator bacteria it is possible to draw the same conclusions, both in terms of potential ability of the inspector to classify carcasses on the basis of visual faecal contamination and in terms of data distribution by sampling point and by carcass weight category. Moreover counts of both *Enterobacteriaceae* and *E. coli* showed variability, not only among slaughterhouses but also among batches slaughtered in the same slaughterhouse, although generally, *E. coli* counts varied the most.

For these reasons, only *Enterobacteriaceae* results are described in the text, while *E. coli* results are available in Appendix I.

2.2.3.1 Comparison of *Enterobacteriaceae* counts on the carcasses categorized in terms of levels of visual faecal contamination.

In Figure 5, *Enterobacteriaceae* counts distribution is reported keeping carcasses classified as dirty or clean as regards the visual contamination *status* separated.

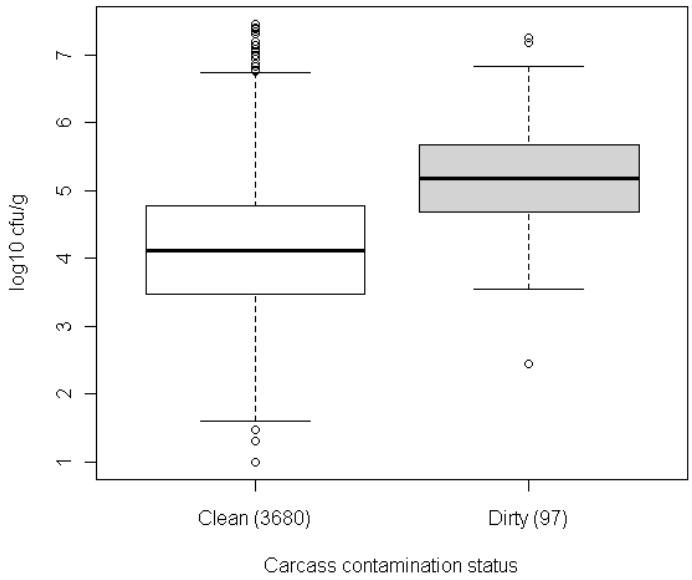


Figure 5: *Enterobacteriaceae* counts ($\log_{10}$ cfu/g) distribution according to the carcass visual cleanliness

In Figure 6, the previous data are expanded keeping the two sampling points separated.

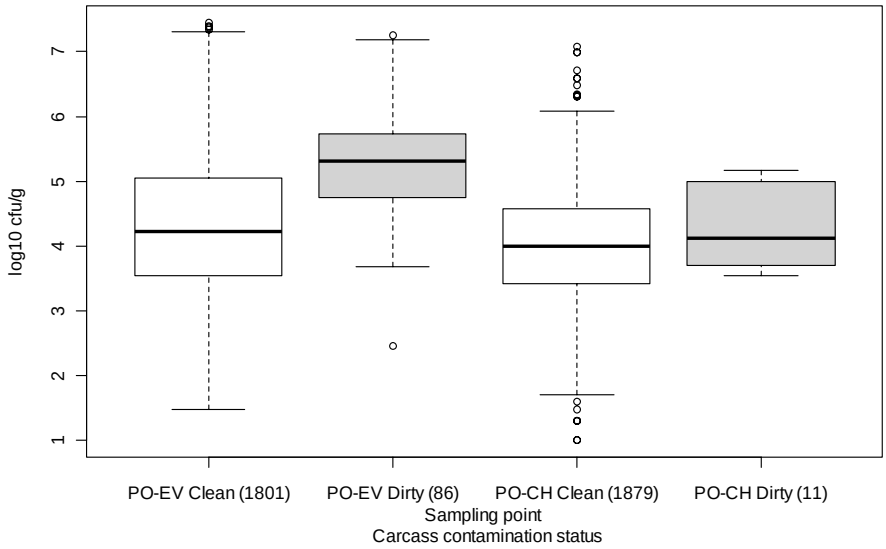


Figure 6: *Enterobacteriaceae* counts (log_{10} cfu/g) distribution according to the carcass contamination *status* at visual inspection according to the sampling point

It is clear that higher *Enterobacteriaceae* counts were recorded in carcasses classified as dirty and that a difference exists between the post evisceration and the post chilling sampling points.

Based on the observed data, the probability of classifying a carcass as a "dirty carcass" both at the post evisceration and post chilling sampling point was estimated for different *Enterobacteriaceae* counts levels defined as "high", where "high value" corresponded to a bacterial load bigger than an arbitrary cut-off value posed, equal to the 70^{th} percentile.

In particular the probability of failure to recognise a carcass as dirty at post evisceration is equal to 88.6%, given an *Enterobacteriaceae* count higher than the 70^{th} percentile (i.e. higher than 4.94 log_{10} cfu/g) (Figure 7).

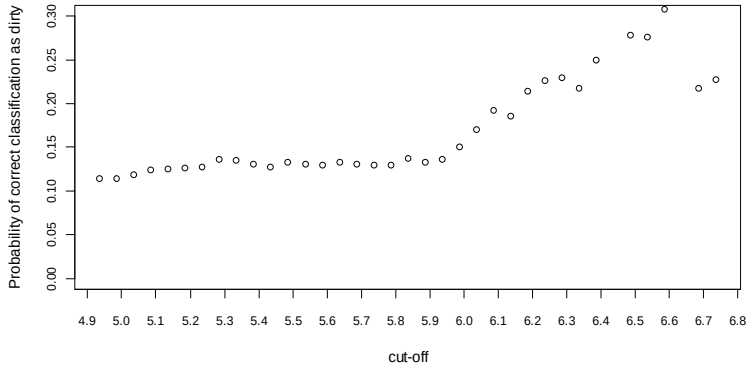


Figure 7: Probability of a carcass being classified as a "dirty carcass" for different *Enterobacteriaceae* cut-off values at the post evisceration sampling point

The probability of failure to recognise a carcass as dirty at post chilling is equal to 99.1%, given an *Enterobacteriaceae* count higher than the 70th percentile (i.e. higher than 4.45 $\log_{10}$ cfu/g) (Figure 8).

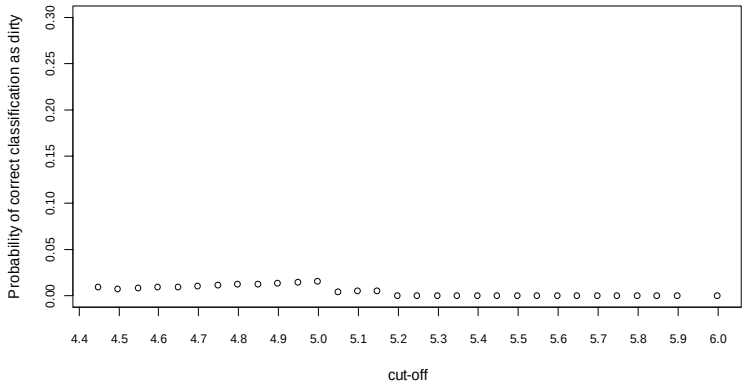


Figure 8: Probability of a carcass being classified as a "dirty carcass" for different *Enterobacteriaceae* cut-off values at the post chilling sampling point

Thus, according to the data collected in this study, the failure in classifying as dirty those carcasses that are considered heavily contaminated (value >70th percentile) with *Enterobacteriaceae* is always higher than 88%, even though it seems to be worse at the post chilling.

2.2.3.2 Data on the variability of the counts of *Enterobacteriaceae* on neck skin of broiler carcasses sampled at post evisceration and post chilling.

In Figure 9, the *Enterobacteriaceae* contamination level is described, keeping the data arising from carcasses collected at post evisceration and post chilling separated.

Generally, higher *Enterobacteriaceae* levels were recorded at post evisceration than at post chilling.

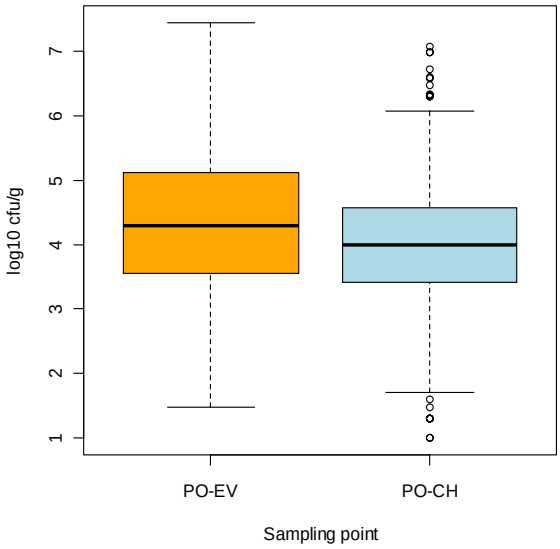


Figure 9: *Enterobacteriaceae* counts (log_{10} cfu/g) distribution according to the sampling point

Enterobacteriaceae counts are summarised in Figure 10, taking into account the distribution of the batches according to the weight category of the broilers.

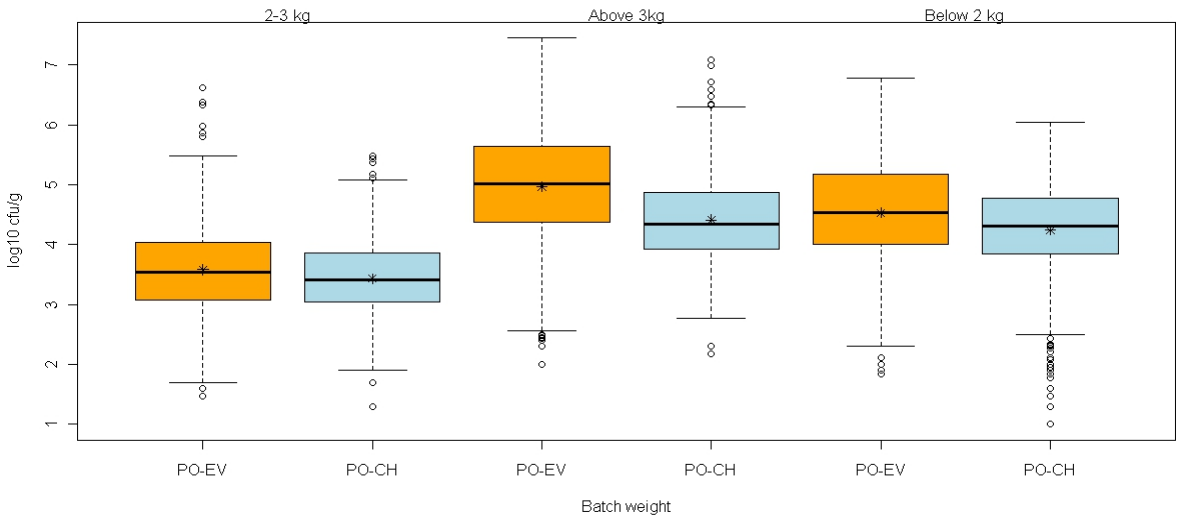


Figure 10: *Enterobacteriaceae* counts (log10 cfu/g) distribution according to the broilers weight category and by the sampling point

Lower levels of *Enterobacteriaceae* tended to occur in carcasses belonging to the weight category 2-3 kg.

In Figures 11 and 12, the distribution of *Enterobacteriaceae* levels recorded in the 7 selected slaughterhouses is described; in Figure 12, counts recorded at the post evisceration and post chilling sampling points are separated.

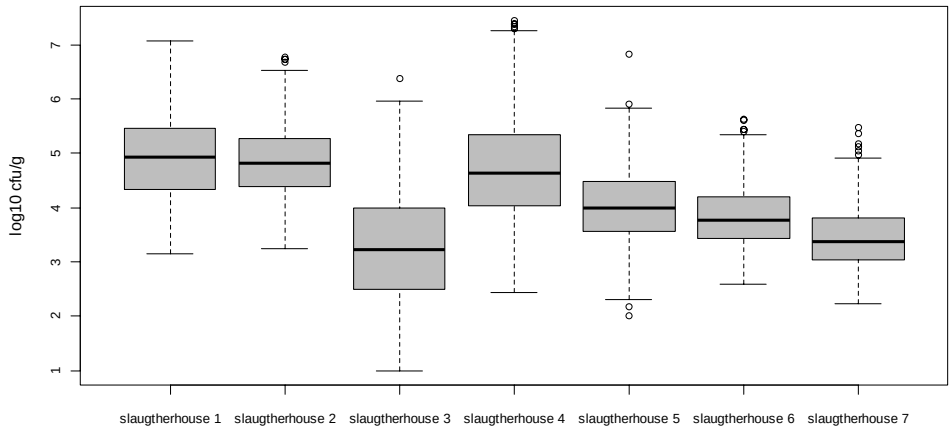


Figure 11: *Enterobacteriaceae* counts (log_{10} cfu/g) in the different slaughterhouses recruited for the study. Both post evisceration and post chilling sampling points are shown.

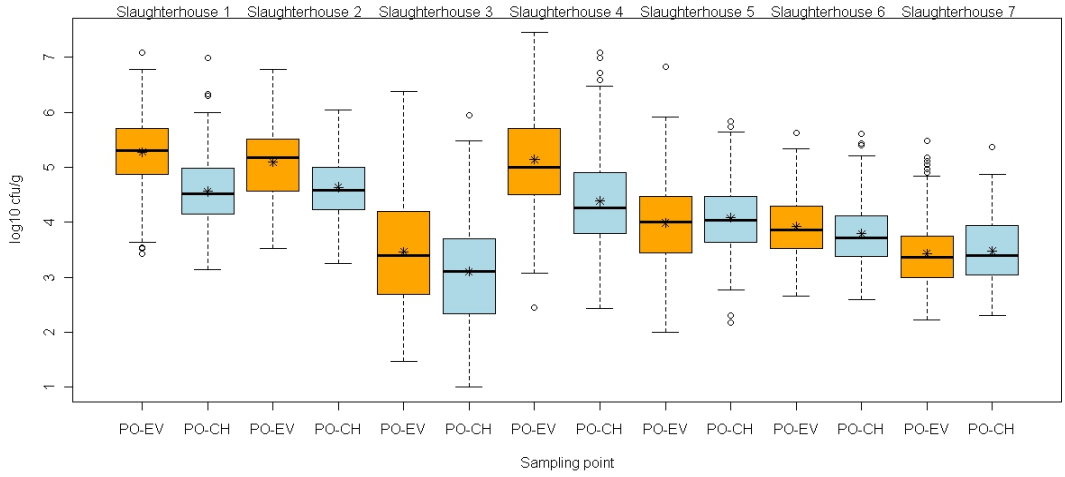


Figure 12: *Enterobacteriaceae* counts (log_{10} cfu/g) in the different slaughterhouses recruited for the study. Both post evisceration and post chilling sampling points are shown. * represents the mean value.

A degree of variability among the slaughterhouses is evident. The mean values are, in most of the cases, higher at the post eviscerations sampling point than at the post chilling sampling point.

In Figures 13 to 26, details as regards *Enterobacteriaceae* counts for each batch within each slaughterhouse are shown. These data show that within the same slaughterhouse, variability exists among batches. Generally, the contamination level at post chilling is lower than at the post evisceration, although some exceptions were observed (Figures 20, 22, and 26).

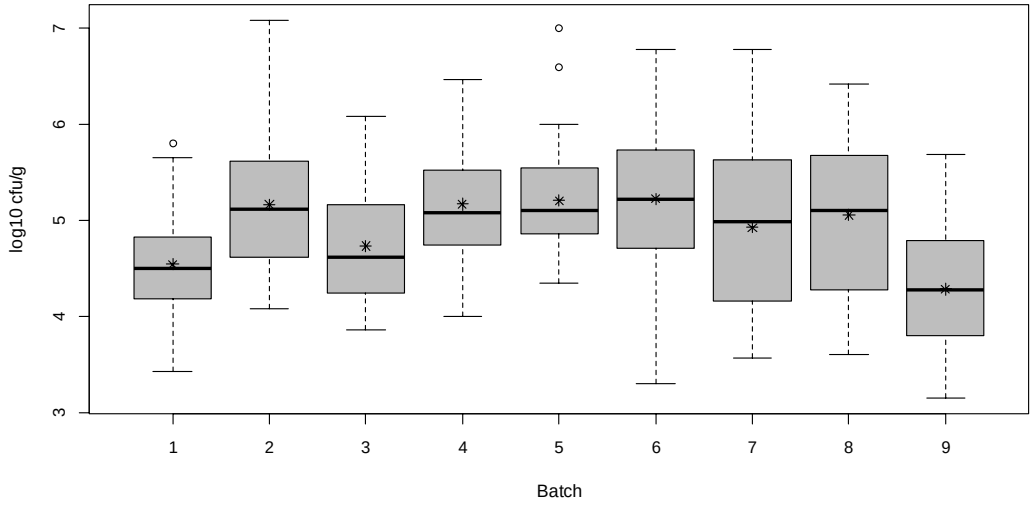


Figure 13: *Enterobacteriaceae* counts (log_{10} cfu/g) distribution per batch for slaughterhouse 1

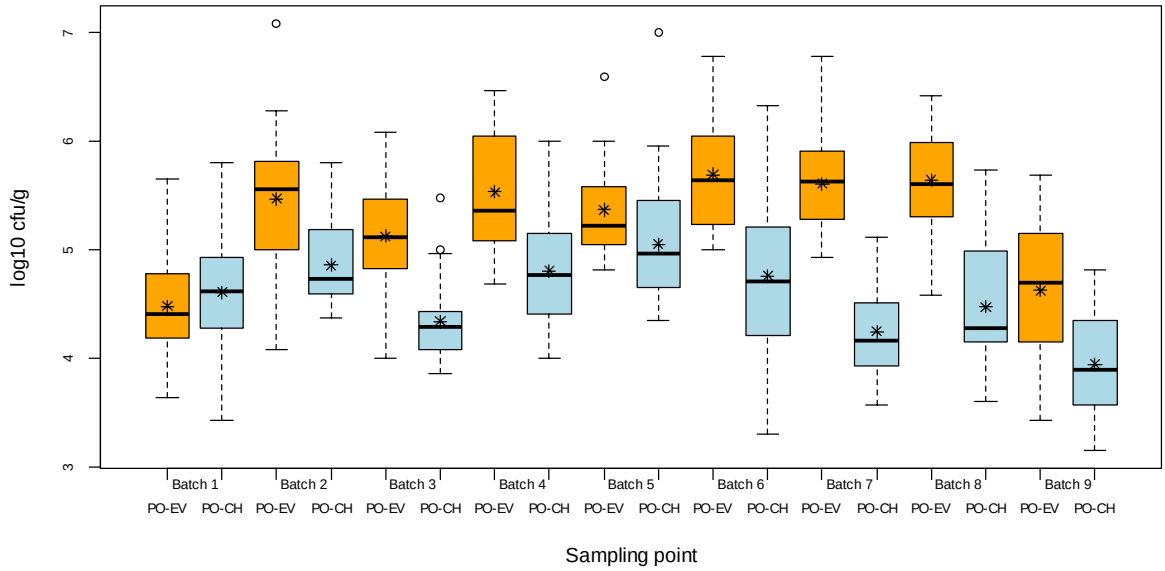


Figure 14: *Enterobacteriaceae* counts (log_{10} cfu/g) distribution per batch and by sampling point for slaughterhouse 1

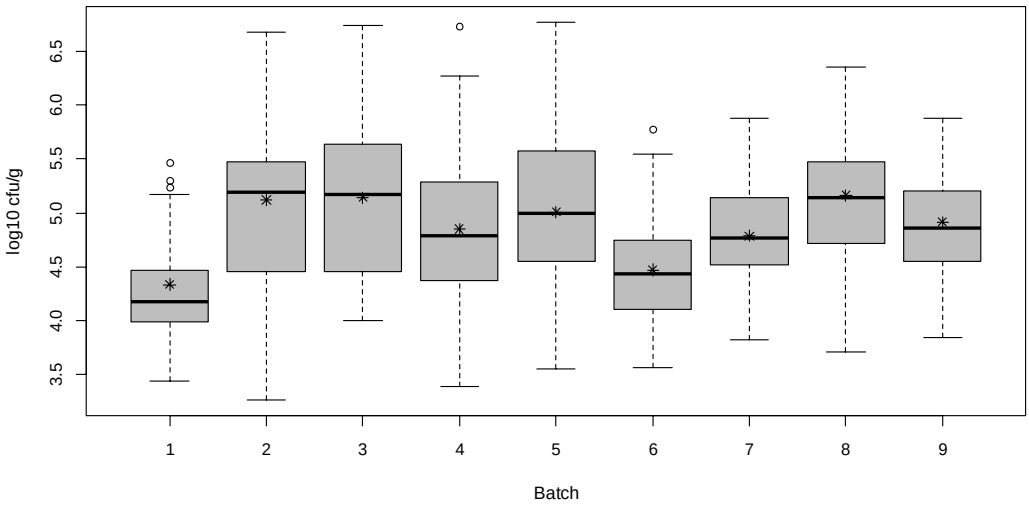


Figure 15: *Enterobacteriaceae* counts (log_{10} cfu/g) distribution per batch for slaughterhouse 2

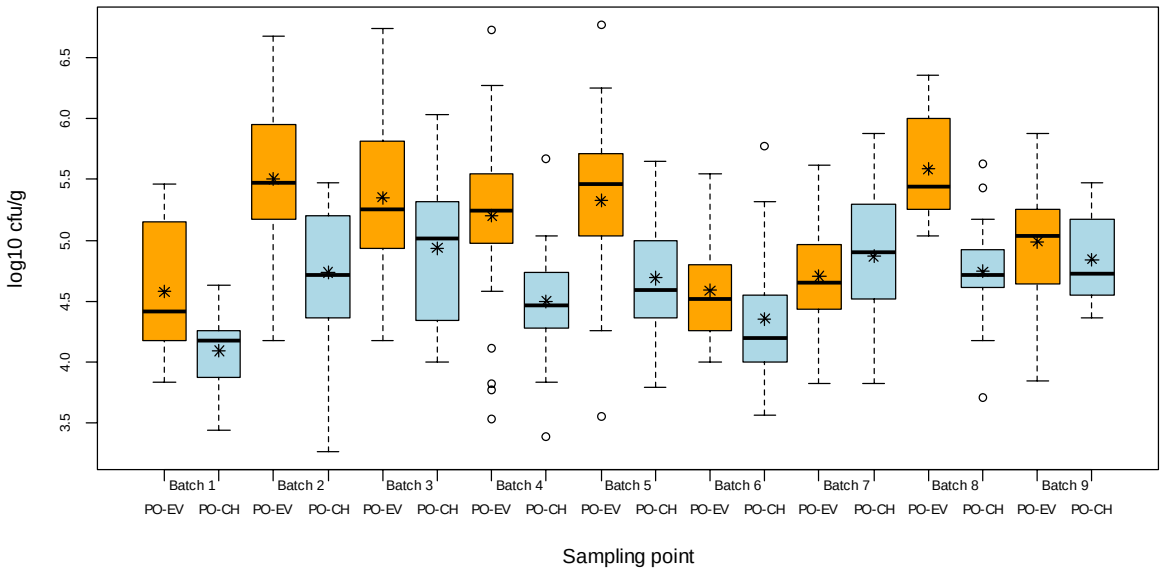


Figure 16: *Enterobacteriaceae* counts (log_{10} cfu/g) distribution per batch and by sampling point for slaughterhouse 2

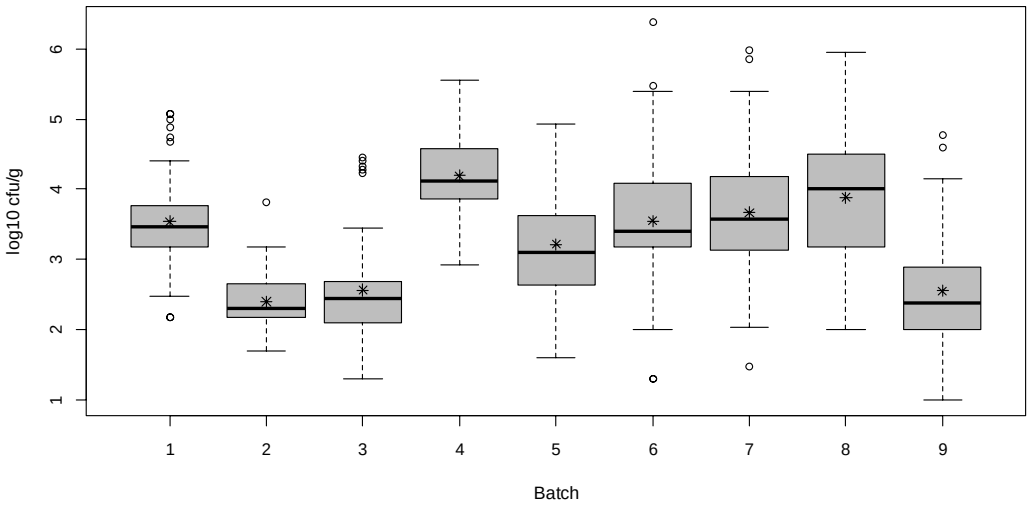


Figure 17: *Enterobacteriaceae* counts (log_{10} cfu/g) distribution per batch for slaughterhouse 3

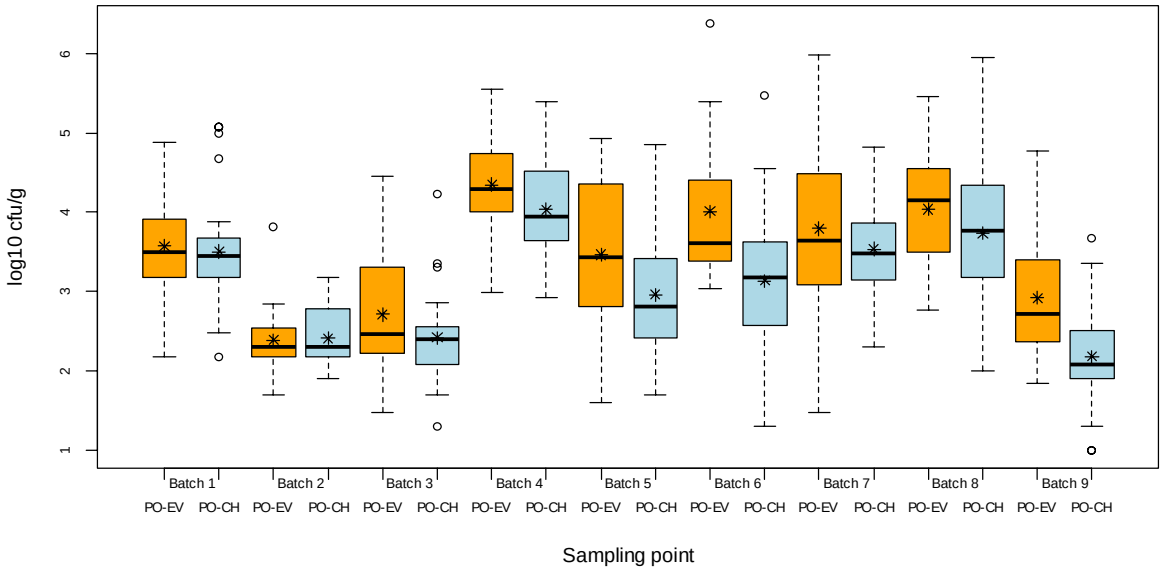


Figure 18: *Enterobacteriaceae* counts (log_{10} cfu/g) distribution per batch and by sampling point for slaughterhouse 3

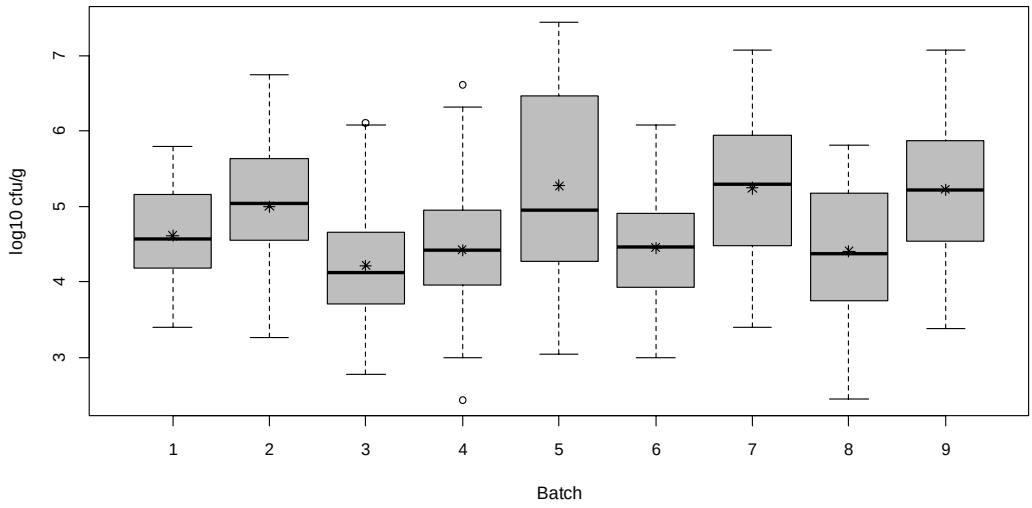


Figure 19: *Enterobacteriaceae* counts (log_{10} cfu/g) distribution per batch for slaughterhouse 4

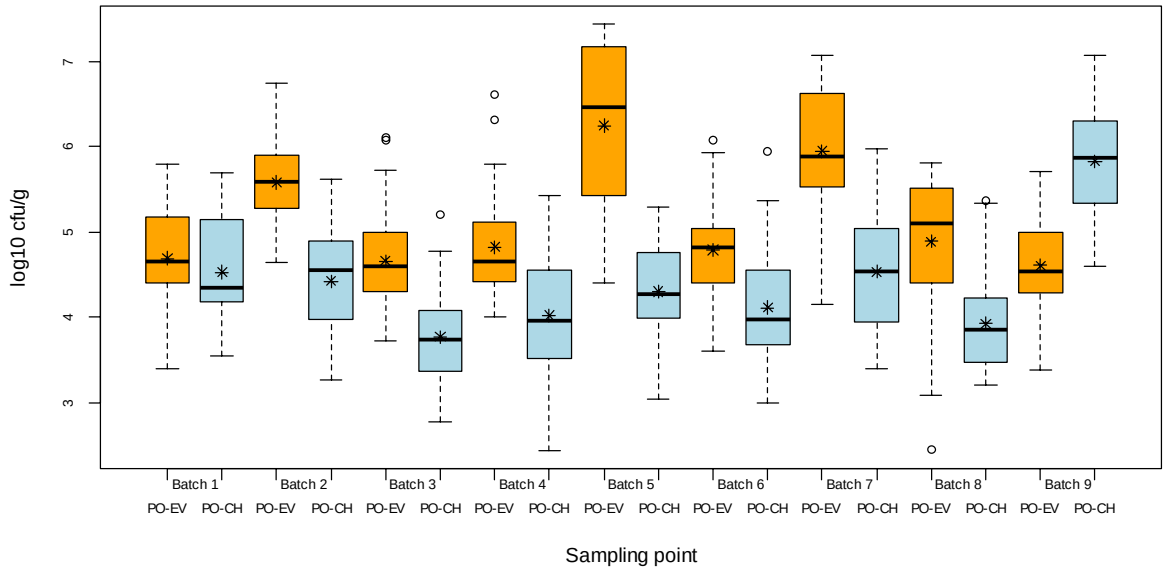


Figure 20: *Enterobacteriaceae* counts (log_{10} cfu/g) distribution per batch and sampling point for slaughterhouse 4

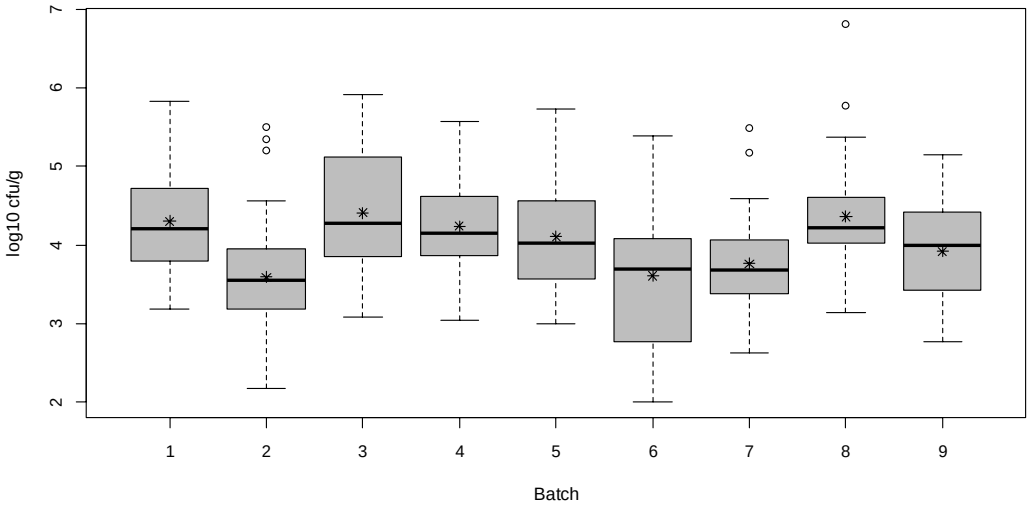


Figure 21: *Enterobacteriaceae* counts ($\log_{10}$ cfu/g) distribution per batch for slaughterhouse 5

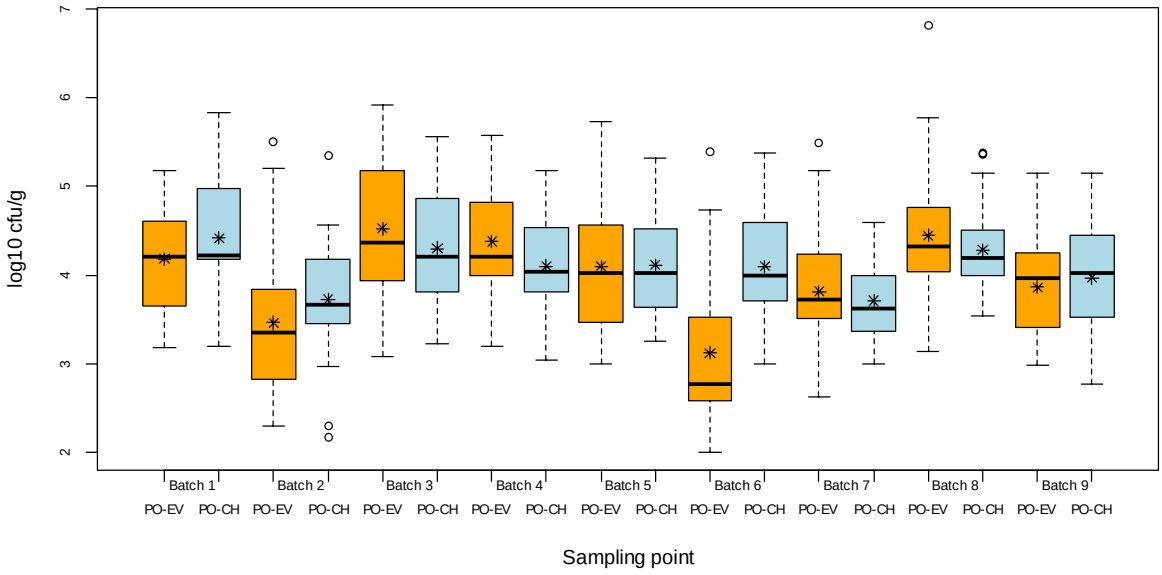


Figure 22: *Enterobacteriaceae* counts ($\log_{10}$ cfu/g) distribution per batch and by sampling point for slaughterhouse 5

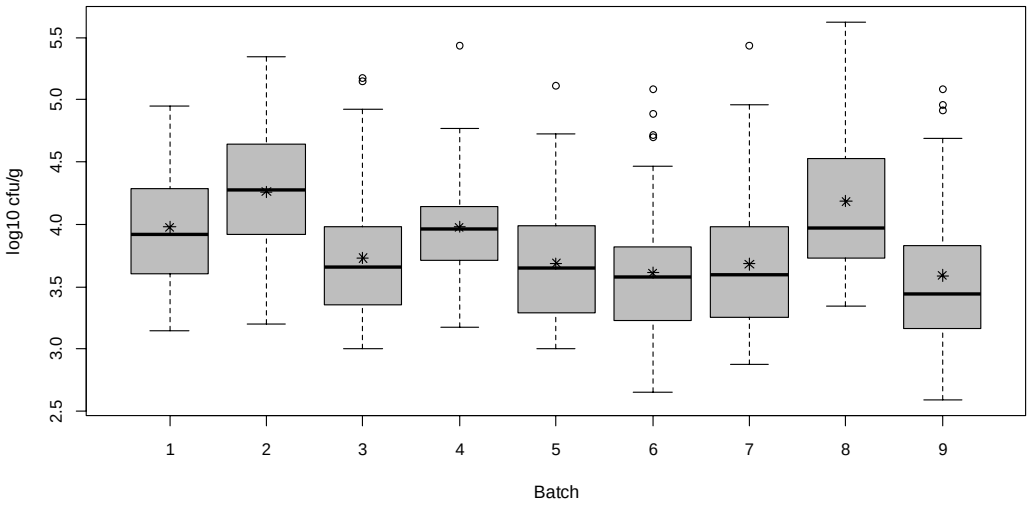


Figure 23: *Enterobacteriaceae* counts (log_{10} cfu/g) distribution per batch for slaughterhouse 6

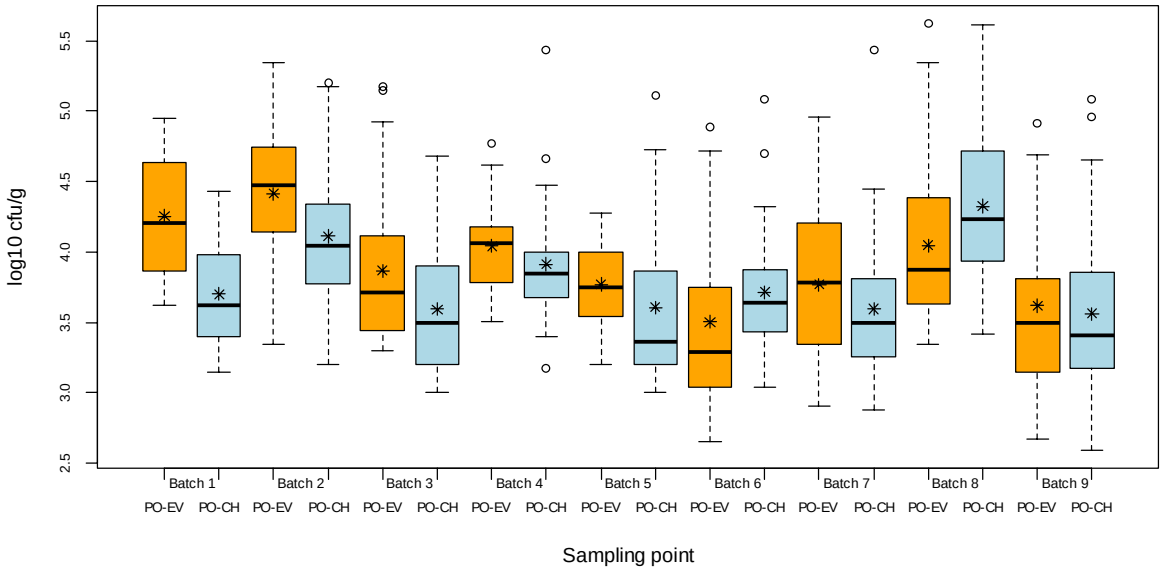


Figure 24: *Enterobacteriaceae* counts (log_{10} cfu/g) distribution per batch and by sampling point for slaughterhouse 6

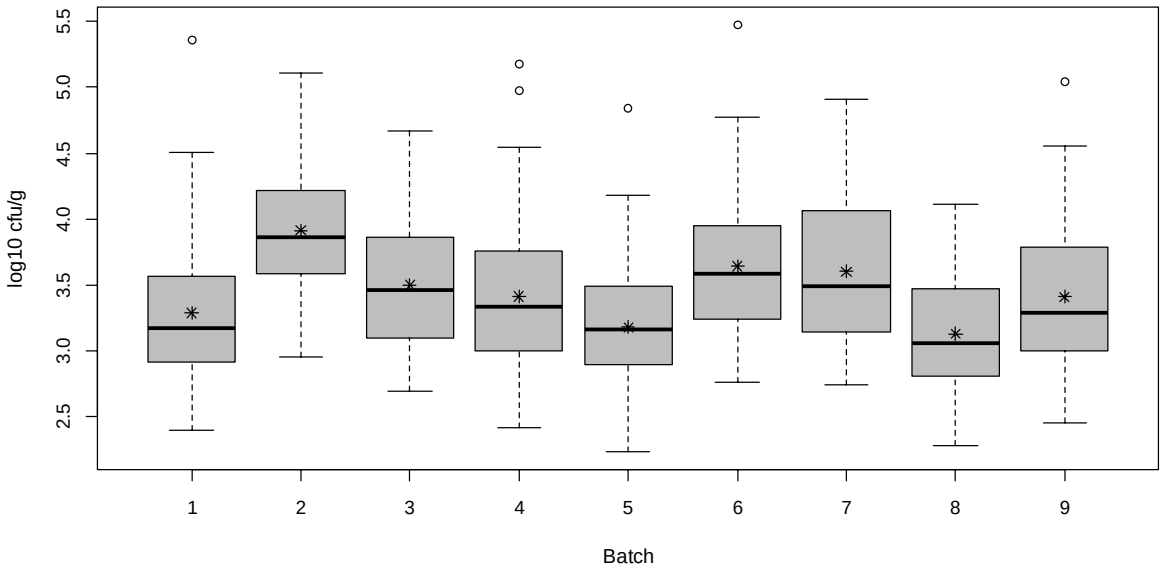


Figure 25: *Enterobacteriaceae* counts (log_{10} cfu/g) distribution per batch for slaughterhouse 7

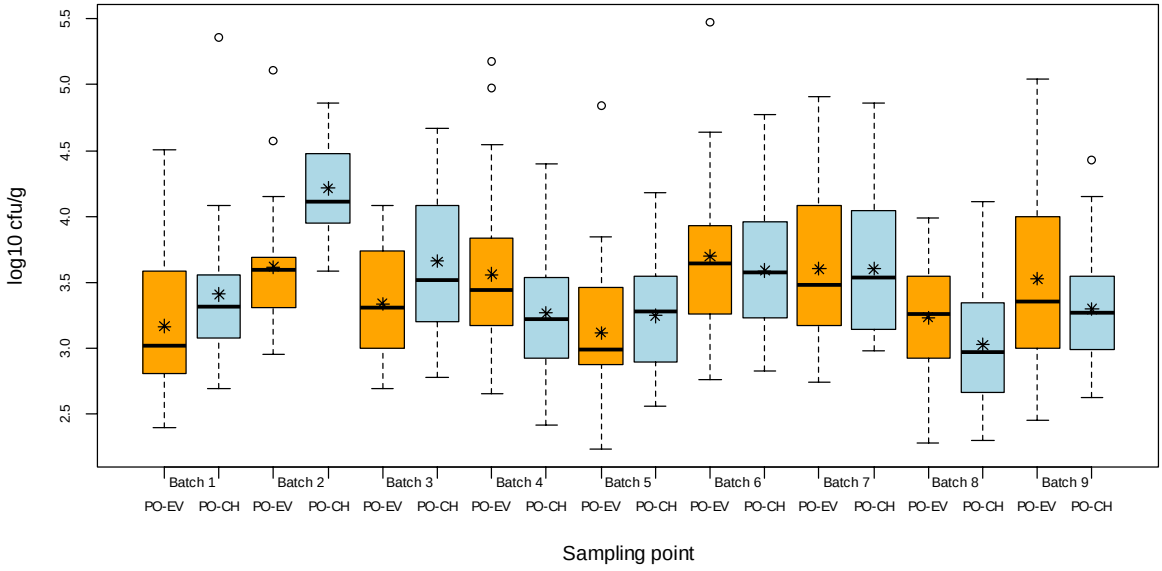


Figure 26: *Enterobacteriaceae* counts (log_{10} cfu/g) distribution per batch and by sampling point for slaughterhouse 7

The possible relationship between *Enterobacteriaceae* counts recorded on carcasses sampled at post evisceration and at post chilling were evaluated, taking into account that, of necessity, different non-corresponding carcasses were sampled at these two sampling points. Thus, the bacterial counts observed on single carcasses at post chilling were related to the average value of the bacterial counts observed on all the sampled carcasses (representative of the batch) at post evisceration.

The graphical representation of the spline function developed for this purpose (Figure 27) shows that a linear relationship can be presumed between counts recorded at the two different sampling points.

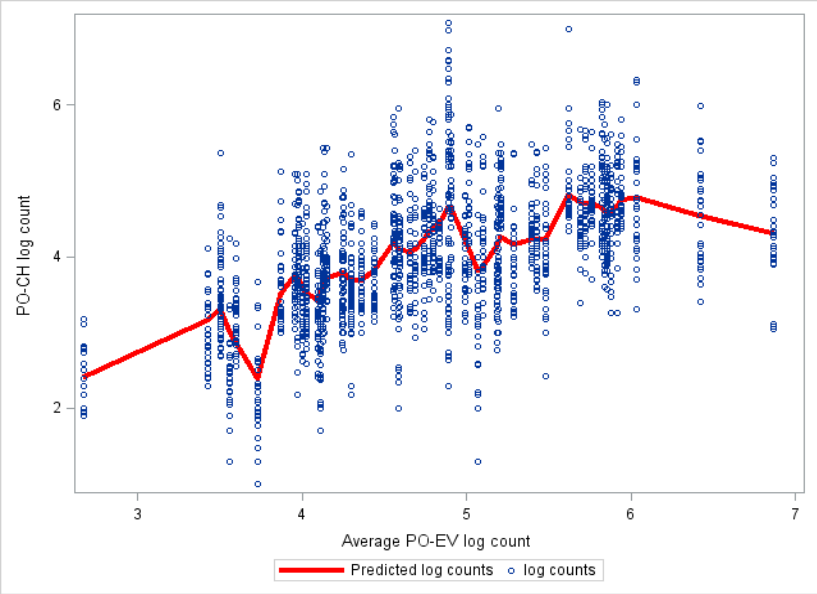


Figure 27: Relationship between *Enterobacteriaceae* carcass counts (log_{10} cfu/g) recorded at the post chilling sampling point and the batch average counts (log_{10} cfu/g) observed at post evisceration sampling point

Finally, in Figure 28, *Enterobacteriaceae* counts distribution is described, keeping batches collected at the beginning of the slaughtering day and toward the end of the slaughtering day separated. From the graph, it appears that the slaughter order does not affect the *Enterobacteriaceae* level on the carcasses.

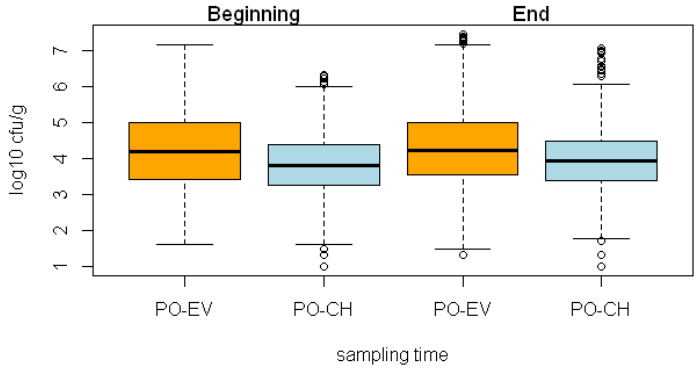


Figure 28: *Enterobacteriaceae* counts (log_{10} cfu/g) distribution according to the time of slaughter

2.3 Statistical model results

In order to verify the data normality that is required by the multilevel mixed linear model, a Q-Q plot was designed considering: all *Enterobacteriaceae/E. coli* counts (Figure 29a/30a), *Enterobacteriaceae/E. coli* counts recorded at post evisceration (Figure 29b/30b) and *Enterobacteriaceae/E. coli* counts recorded at post chilling (Figure 29c/30c).

Figures 29 and 30 show the Q-Q plots. The graphs support the assumption of data normality distribution which is required by the statistical model.

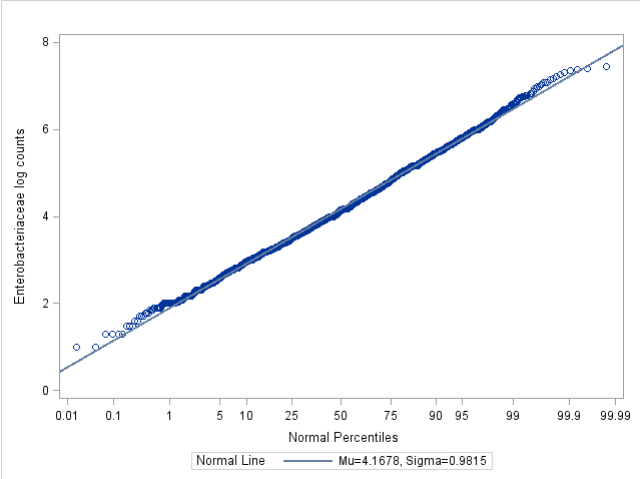
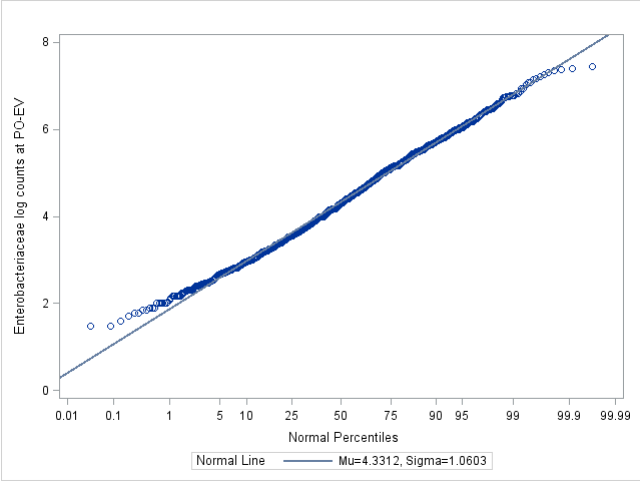


Figure 29a: Q-Q plots for all *Enterobacteriaceae* counts (log_{10} cfu/g)



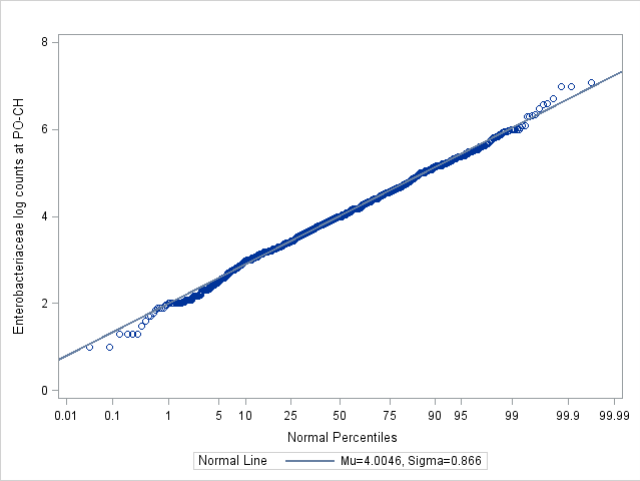


Figure 29 b, c: Q-Q plots for *Enterobacteriaceae* counts (log_{10} cfu/g) recorded at the post evisceration (left) and at the post chilling (right) sampling points.

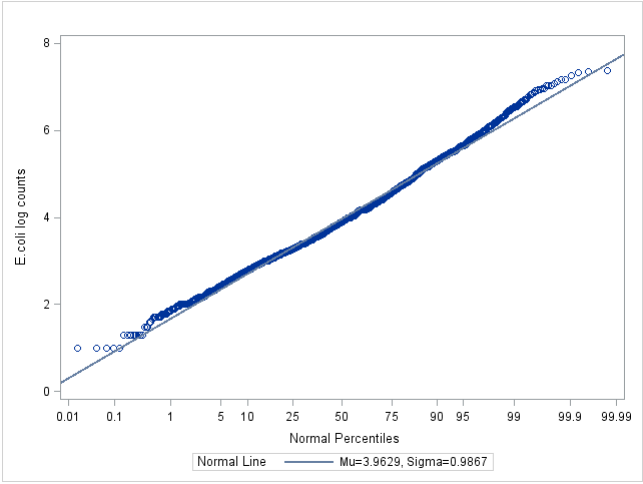


Figure 30a: Q-Q plots for all *E. coli* counts (log_{10} cfu/g)

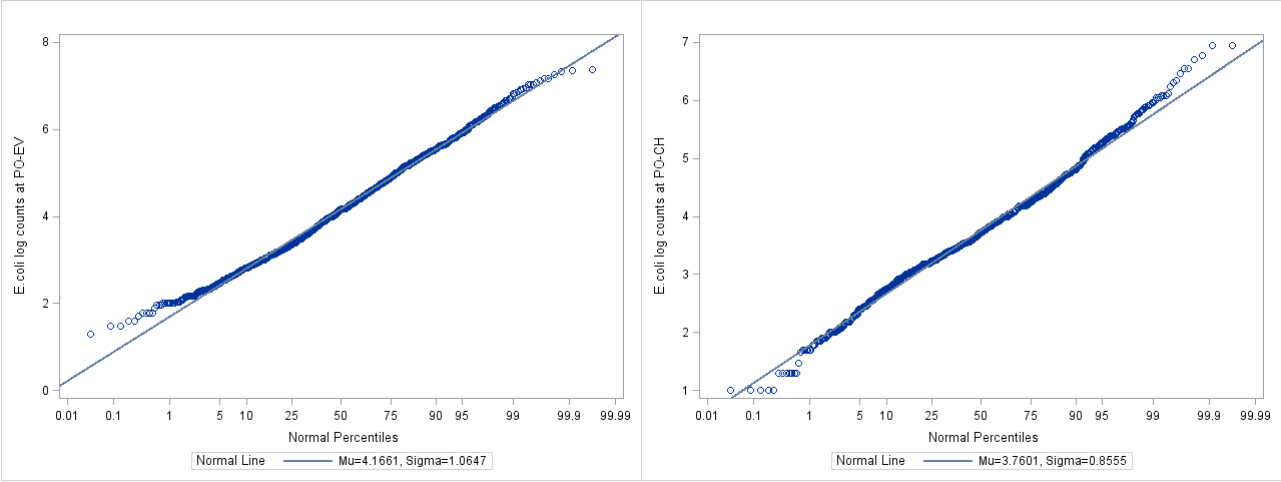


Figure 30 b, c: Q-Q plots for E. coli counts (log10 cfu/g) recorded at the post evisceration (left) and at the post chilling (right) sampling points.

2.3.1 Variables considered in the models

All the variables used in the models originating from the questionnaires and the carcass form are reported in Table 4 (a, b, c) where for each variable the short name and its meaning are described and details are provided on the type of variable.

Table 4a: Slaughterhouse (level 3) variables included in the models: the short name, the description of the variables and additional notes are reported

Variable short name	notes	description of the variable
capacityBroilers		Broilers slaughtered per year
throughputBroiler	c	Broilers slaughtered per day (average)
throughputBatches	c	Batches slaughtered per day (average)
workingHours	c	Working hours per day (average)
workingDays	c	Working days per week (average)
Linespeed	b, c	Lines speed for broilers weight category (number of broilers per minute)
processingTime	b, c	Time between stunning and beginning of chilling for broilers weight category (minutes)
noOperators	c	Number of operators at the slaughter line/s
shiftHrs	c	Work shift day (hours)
singleRoom		Slaughter line in a single room (presence/absence)
noLines		Number of slaughter lines
stunningType		Type of stunning
bathType		Scalding method
scaldingTemp	c	Average scalding temperature
scaldingTime	b, c	Time of scalding for broilers weight category (minutes)
defeathering		Plucking method
manualPluck		Presence/absence of manual plucking
washPostPluck		Presence/absence of washing post plucking
evisceration		Type of evisceration
inspectionPoint		Point of inspection
washPostEvis*		Presence/absence of washing post evisceration
washDecont*		Presence/absence of decontamination washing
chillingTime	b, c	Time of chilling phase for broilers weight category (minutes)
chillingTech*		Chilling technique
chillingTemOmo*		Presence/absence of homogenous chilling temperature
meanchillingTemp*	n	Average temperature of chilling
refrigerationRoom*		Presence/absence of a refrigeration room
freqCleaning		Frequency of slaughterhouse cleaning

c: continuous variable
*: variable considered only in the M3 and M4 models
n: new variable, not present in the questionnaires.
b: variable used at batch level considering the broilers weight

Table 4b: Batch (level 2) variables included in the models: the short name, the description of the variables and additional notes are reported

Variable short name	notes	description of the variable
sampTemp	c	Outside temperature on the sampling day
sampTime		Time of sampling
min_delta_loading	c, n	Time for loading (minutes)
weather		Weather conditions on the sampling day
weather_2	r	Weather conditions on the sampling day
N_batch	c	Number of broilers in the sampled batch
batchType		Type of batch (entire flock or part of a flock)
N_DOA	c	Number of broilers in the sampled batch
N_DOA_2	r	Number of broilers in the sampled batch in categories
age	c	Age of the broilers (days)
batchWeight		Wight category of the broilers in the sampled batch
crate_m2	c, n	Number of broilers per crate on average /surface area per crate (m^2)
min_delta_sla	c, n	Time for slaughter (minutes)
tempHoldPenp	c	Temperature at the holding pens (°C)
p_rejects	c	Number of discarded animals per batch at *post mortem*
p_rejects_01	r	Presence/absence of rejected animals
lesioFarm		Type of lesions due to farm management
PctIntestRupture	c	% of intestinal rupture
PctIntestRupture_01	r	Presence/absence of intestinal rupture
extTempPostEvis	c	EXTERNAL temperature of one carcass after evisceration
extTemp*	c	EXTERNAL temperature of one carcass after chilling
intTemp*	c	INTERNAL temperature of one carcass after chilling
deltaTemp*	c, n	Difference between EXTERNAL temperature PO/EV and PO/CH
faecalContamAMStudy		*Ante mortem* classification on the base of faecal contamination level according to study criteria
timetempchilling*	c, n	Time X Temperature of chilling
batchWeight*sampInfo	n	Interaction between weight category and sampling point
log_counts_POEV	n	Average value of log counts at PO-EV
log_counts_POEV* batchWeight	n	Interaction between average value of log counts at PO-EV and weight category

c: continuous variable
*: variable considered only in the M3 and M4 models
r: reclassified variable (variable present in the questionnaire but modified for the analysis)
n: new variable, not present in the questionnaires

Table 4c: Carcass (level 1) variables included in the models: the short name, the description of the variables and additional notes are reported

Variable short name	notes	description of the variable
sampMatInfo		Visual evaluation of carcasses (clean/dirty)
sampInfo		Sampling point (post evisceration /post chilling)
sampMatInfo*sampInfo	n	Interaction between visual evaluation of carcasses and sampling point

*: variable considered only in the M3 and M4 models
n: new variable, not present in the questionnaires.

At the slaughterhouse level, the variables "killing method", "occurrence of leakage during evisceration" and "chilling method" were not included in the models for absence of variability; the variables "washing between the evisceration and the inspection point", "decontamination washing water temperature" and "type of washing decontamination" were not included in the models because information was not provided.

Since the sampled batches in those slaughterhouses that declared they plan their slaughter according to *Salmonella status* were all negative, variables related to planning of slaughter on the basis of the sanitary *status* of the flock were not included in the models.

At the batch level, the variables "production method", "homogeneity of the animal weight within the batch", "information on test for *Salmonella"* and "presence of feed in the crop" were not included in the models for absence of variability; while, the variables "time of catching", "information on test for *Campylobacter",* "duration of feed withdrawal" and "presence of lesions due to the slaughtering" were not included in the models because information was not provided.

Further details on data availability are reported in the descriptive statistical analysis results.

2.3.2 Models' outputs

The outputs of the models are described according to the approach that is reported in materials and methods. First, the evaluation on the structure of the hierarchical model is described (Tables 5 and 6). Secondly, the models' outputs using models 1 to 4 (M1 to M4) are summarised in Table 6.

Basically, each model was applied both to *E. coli* and *Enterobacteriaceae*; the possible different scenarios within each main model, on the basis of the variables that turned out to be significant each time are highlighted.

Step 1: In Table 5, the likelihood values of the models with a fixed intercept, random effects associated with the intercept for batches (Level 2) and slaughterhouses (Level 3), and of the model with a fixed intercept, random effects associated with slaughterhouses (Level 3), are reported. Moreover, the likelihood values calculated for each model (from M1 to M4) and indicator bacteria (*E. coli* and *Enterobacteriaceae*) are shown. Results of the statistical test $\chi^2_{(0:1)}$ and the associated p-values are reported.

Table 5: Evaluation of the structure of the hierarchical model

		Likelihood three level models ($\sigma^2_{\text{int:slaugh}}$; $\sigma^2_{\text{int:batch}}$)	Likelihood two level models ($\sigma^2_{\text{int:slaugh}}$)	$\chi^2_{(0:1)}$	p-value
M 1	*E. coli*	8282.90	8963.5	680.6	<0.0001
	Enterobacteriaceae	7828.9	8590.9	762	<0.0001
M 2	*E. coli*	3849.6	4346.8	497.2	<0.0001
	Enterobacteriaceae	3698	4293.9	595.9	<0.0001
M 3	*E. coli*	3604.9	4157.4	552.5	<0.0001
	Enterobacteriaceae	3391	3957.8	566.8	<0.0001
M 4	*E. coli*	3604.9	4157.4	552.5	<0.0001
	Enterobacteriaceae	3391	3957.8	566.8	<0.0001

The $\chi^2_{(0:1)}$ test indicates that including the random effects for batch is appropriate. Thus, all models (M1 to M4) include both the slaughterhouse and the batch intercept random effects.

Table 6 reports the values of random effects variance associated with the intercept for slaughterhouses, for batches and residuals. The value of the relative ICC_{slaugh} and ICC_{batch} are shown.

Table 6: Values of random effects variance and ICC

		$\sigma^2_{\text{int:slaugh}}$		$\sigma^2_{\text{int:batch}}$		σ^2			
		Estimate	Standard Error	Estimate	Standard Error	Estimate	Standard Error	ICC_{slaugh}	ICC_{batch}
M1	*E. coli*	0.3952	0.2377	0.1397	0.02796	0.4971	0.01154	0.382946	0.518314
	Enterobacteriaceae	0.4519	0.2702	0.138	0.02747	0.4399	0.01021	0.438823	0.57283
M2	*E. coli*	0.6314	0.3775	0.1876	0.03801	0.4065	0.01346	0.515218	0.668299
	Enterobacteriaceae	0.6365	0.3816	0.2073	0.04153	0.3731	0.01235	0.52305	0.693401
M3	*E. coli*	0.229	0.1448	0.1824	0.03672	0.3559	0.01178	0.298449	0.536166
	Enterobacteriaceae	0.3131	0.1922	0.1669	0.03354	0.3172	0.01049	0.39275	0.602107
M4	*E. coli*	0.229	0.1448	0.1824	0.03672	0.3559	0.01178	0.298449	0.536166
	Enterobacteriaceae	0.3131	0.1922	0.1669	0.03354	0.3172	0.01049	0.39275	0.602107

The random effects variance and the value of intra-class correlation show that the proposed model structure is plausible.

In all proposed models that consider only the intercept and the random effects, the value of ICC_{slaugh} is high, and this means that the total random variation is dominated by the variance of the random slaughterhouse effects. In other words, the ICC_{slaugh} is high if the log_{10} counts of carcasses in the same slaughterhouse are relatively homogeneous and at the same time the log_{10} counts across slaughterhouses tend to vary widely.

Also, the value of ICC_{batch} is high and this means that there is little variation in the log_{10} counts of carcasses within the same batch compared to the total random variation.

The ICC_{batch} values are always higher compared to the ICC_{slaugh} values, suggesting that a higher homogeneity of observed counts exists within the same batch than within the same slaughterhouse.

Steps from 2 to 5: In Table 7, results, in terms of variables which proved to be significant in the different proposed models (M1 to M4) are reported.

Variables of fixed effects that affect the *E. coli* and *Enterobacteriaceae* counts are grouped according to the hierarchical structure.

In all cases, the residual analysis showed that Studentized conditional and marginal residuals satisfied the condition of goodness of fit of the models (Figures from 1 to 14 and 16, 17, 19, 20 reported in Appendix J).

Table 7: Significant variables for each model considered

				M1		M2			M3		M4	
		Variables	General notation	C	E	C	C*	E	C	E	C	E
	Level	Intercept	β_0	✓	✓	✓	✓	✓	✓	✓	✓	✓
Fixed effect	Carcass	Visual evaluation of carcasses (clean/dirty)	β_1	✓	✓	✓	✓	✓				
		Sampling point (PO-EV; PO-CH)	β_2	✓	✓	∘	∘	∘	∘	∘	∘	∘
		Interaction between visual evaluation and sampling point	β_3	✓	✓	∘	∘	∘	∘	∘	∘	∘
	Batch	Weight category of the broilers in the sampled batch	β_4	✓	✓	✓	✓	✓	✓	✓	✓	✓
		Interaction between weight category and sampling point	β_5	✓	✓							
		Presence/absence of intestinal rupture	β_6			✓						
		Presence/absence of rejected animals	β_7						✓	✓		
		Average value of log counts at PO-EV	β_8			∘	∘	∘	∘	∘	✓	✓
		Interaction between average value of log counts at PO-EV and weight category	β_9			∘	∘	∘	∘	∘	✓	✓
	Slaughterhouse	Plucking method	β_{10}		✓		✓	✓				
Random effect	Batch (*j*)	Intercept	$u_{i/j}$	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Slaughterhouse (*k*)	Intercept	u_k	✓	✓	✓	✓	✓	✓	✓	✓	✓
Residuals	Carcasses (*i*)		ε_{ijk}	✓	✓	✓	✓	✓	✓	✓	✓	✓
Covariance parameters for G matrix	Batch level	Variance of intercept	$\sigma^2_{\text{int: batch}}$	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Slaughterhouse level	Variance of intercept	$\sigma^2_{\text{int: slaugh}}$	✓	✓	✓	✓	✓	✓	✓	✓	✓
Covariance parameters for R matrix	Carcasses level	Residual variance	σ^2	✓	✓	✓	✓	✓	✓	✓	✓	✓

C: Model for *E. coli* $\log_{10}$ counts
E: Model for *Enterobacteriaceae* $\log_{10}$ counts
*: Alternative scenario
✓: Significant variables
∘: Variables that cannot be included in the model
Empty cells: variables not significant

M1 results

The aim of model 1 was to evaluate which variables at carcass, batch and slaughterhouse level (excluding variables related to the slaughtering phases following evisceration) significantly affect *E. coli* or *Enterobacteriaceae* counts considering carcasses sampled at post evisceration and post chilling sampling points.

M1, which takes into account all the observed counts, demonstrates that, at carcass level, the visual evaluation of carcasses (the fact that carcasses were classified as "dirty" or as "clean"), the sampling point (the fact that carcasses were sampled at post evisceration or at post chilling), and the interaction between these two variables are significant in explaining the variability of counts (Table 7 and Tables 2 and 3 of Appendix J).

In particular, the bacterial levels are significantly higher in carcasses sampled at the post evisceration compared to those sampled at the post chilling sampling point and in dirty carcasses compared to clean ones. As regards this last point, the significance is observed for *E. coli* but not for *Enterobacteriaceae* (Table 22), although the interaction between these two variables is significant for both indicators. The significance of the interaction underlines that the difference related to the sampling points is independent from the contamination *status* identified through the visual inspection, whereas the effect of the visual evaluation is relevant only at the evisceration point (Tables 2 and 3 reported in Appendix J).

At batch level, the weight of the carcasses within the batch (weight category of the batch) and the interaction between batch weight category (level 2 variable) and sampling point (level 1 variable) are the only two significant variables (Table 7 and Tables 2 and 3 of Appendix J). The bacterial levels identified in carcasses belonging to batches "between 2-3 kg" weight category are significantly lower compared to those belonging to batches "below 2 kg" and "above 3 kg" weight categories at the post evisceration sampling point. At the chilling sampling point, this significant difference is no longer evident between 2-3 kg and above 3 kg weight categories but is maintained between the 2-3 kg and below 2 kg weight categories. At both sampling points, no difference exists between $\log_{10}$ counts recorded in carcasses belonging to the lowest and the highest batches weight category (Tables 2 and 4 reported in Appendix J).

Whereas for *E. coli,* no variables are significant at slaughterhouse level, for *Enterobacteriaceae*, the plucking method significantly affects the contamination level (Table 7; Table 2 reported in Appendix J). The result is that the *Enterobacteriaceae* $\log_{10}$ counts are lower when the plucking method used is the vertical disk compared to the combined techniques (vertical, horizontal and counter-rotating). No significant difference exists between vertical or horizontal disk (Table 4 reported in Appendix J).

M2 results

The aim of model 2 was to evaluate which variables at carcass, batch and slaughterhouse level (excluding variables related to the slaughtering phases following evisceration) significantly affect *E. coli* or *Enterobacteriaceae* counts, focusing on carcasses sampled at the post evisceration sampling point.

M2, which takes into account only the $\log_{10}$ counts observed at the post evisceration sampling point, includes two different scenarios in terms of variables which proved to be significant as regards *E. coli* counts; the second of these two scenarios also affected *Enterobacteriaceae* counts.

As regards the first scenario, at carcass level (level 1), only the variable "visual evaluation of carcasses" could be included, and it proved to be significant (confirming the results obtained by M1), (Table 7; Table 5 reported in Appendix J). Specifically, the bacterial loads detected in carcasses classified as dirty are significantly higher than those detected in carcasses classified as clean (Table 12 reported in J). At batch level (level 2), the weight category and the presence of intestinal ruptures are the variables that proved to be significant (Table 7; Table 5 reported in Appendix J). In particular, batches with broilers weight of 2-3 kg are contaminated with lower *E. coli* levels compared to batches below 2 kg, and batches without intestinal ruptures have lower *E. coli* $\log_{10}$ counts compared to batches with intestinal ruptures (Table 6 reported in Appendix J). No variables at the slaughterhouse level (level 1) were significant (Table 7; Tables 5 and 6 reported in Appendix J).

The second scenario, which fitted for both types of indicator bacteria, allowed the identification of the following variables as significant: "visual evaluation of carcasses" at carcass level (level 1), "weight category" at batch level (level 2) and the "plucking method" at slaughterhouse level (level 3) (Table 7; Tables 7 and 9 reported in the Appendix J). The conclusions are the same as those presented by M1, but in this case a reduction of importance of the variable "weight category" is verified; in fact the F test of fixed effect (Tables 7 and 9 reported in Appendix J) shows that the weight is not so important in this scenario although the counts recorded in batches with broilers of 2-3 kg are lower compared to those recorded in batches of the other two weight categories (Tables 8 and 10 reported in Appendix J). As regards the plucking method, the $\log_{10}$ counts of the indicator bacteria are lower when the plucking method used is the vertical or horizontal disk compared to the combined techniques. No significant difference exists between vertical or horizontal disk only (Tables 8 and 10 reported in Appendix J).

To conclude, the BIC value indicates that this second scenario is preferable to explain the variability of *E. coli* counts (Tables 5 and 7 reported in Appendix J).

M3 results

The aim of model 3 was to evaluate which variables at carcass, batch and slaughterhouse level (all variables included) significantly affect *E. coli* and *Enterobacteriaceae* counts, focusing on carcasses sampled at the post chilling sampling point.

In this model, at carcass level (level 1) only the variable "visual evaluation of carcasses" was considered, but it proved to be not significant (confirming the results obtained by M1) (Table 7; Tables 11 and 13 reported in Appendix J).

At batch level (level 2), the weight of carcasses and the presence of discarded animals at *post mortem* were the significant variables (Table 7; Tables 11 and 13 reported in Appendix J). In particular, batches of 2-3 kg had lower bacterial levels compared to batches below 2 kg and above 3 kg, and batches without discarded animals show lower $\log_{10}$ counts compared to batches with discarded animals (Tables 12 and 13 reported in Appendix J).

No variables at slaughterhouse level (level 3) were significant (Table 7; Tables 11 and 13 reported in Appendix J).

It is important to underline that including the variable "presence of discarded animals" at batch level or the variable "stunning type" or "chilling technique" at slaughterhouse level resulted in the same value of model's likelihood.

An explanation for this result is that the slaughterhouse that presented the lowest bacterial counts was also the only one that did not have rejected carcasses (slaughterhouse 3). Further, this slaughterhouse was the only one that used the electronarcosis method (vs. the electronarcosis in water) to stun the birds, and to use a refrigeration room (vs. a tunnel) as the chilling technique. Thus, it is very difficult to attribute the low bacterial levels to the absence of discarded animals or to one or more slaughter techniques adopted in the slaughterhouse.

According to the hierarchical structure of these types of models and the criteria used in this study, which first consider the variables at batch level and afterwards the variables at slaughterhouse level, the variable "presence of discarded animals" is the one that was accepted in the final version of M3.

M4 results

The aim of model 4 was to evaluate which variables at carcass, batch and slaughterhouse level (all variables included) significantly affect *E. coli* and *Enterobacteriaceae* counts, focusing on carcasses sampled at the post chilling sampling point. Additionally to M3, a new variable at batch level was created, corresponding to the average value of counts recorded on carcasses collected at post evisceration, in order to highlight the relationship between bacterial loads at post evisceration and bacterial loads at post chilling (variable at level 1).

In M4, at carcass level (level 1), only the variable "visual evaluation of carcasses" can be considered, but it proved to be not significant (confirming the results obtained by M1) (Table 7; Tables 15 and 17 reported in Appendix J).

At batch level (level 2), the weight category, the average $\log_{10}$ counts recorded on carcasses collected at the post evisceration sampling point and their interaction proved to be significant (Table 7; Tables 15 and 17 reported in Appendix J). In particular, the average level of counts positively influences the $\log_{10}$ bacterial count values at the post chilling sampling point. This effect is more evident for the batches with broilers below 2 kg and 2-3 kg compared to the batches with broilers of greater weight (Figures 15 and 18 reported in Appendix J).

Given that the average values of the counts recorded on the carcasses at the post evisceration sampling point were 4.67 cfu $\log_{10}$/g and 4.77 cfu $\log_{10}$/g for *E. coli* and *Enterobacteriaceae,* respectively, the estimated average bacterial counts for broilers in the 2-3 kg weight batches proved to be significantly lower than those estimated for the other two weight categories. No significant difference exists between lower and higher batch weight categories (Tables 16 and 18 reported in Appendix J).

3. CONCLUSIONS

A sampling campaign was carried out in seven poultry slaughterhouses located both in Denmark and Italy and considered to be illustrative of European slaughterhouses. The sampling plan, performed from April to the beginning of September 2013, allowed the quantification of the level of *E. coli* and *Enterobacteriaceae* in 3 777 samples of broiler neck skin, 1 887 obtained from carcasses at post evisceration and 1 890 obtained from carcasses at post chilling (sixty carcasses per batch were collected except in one case). Out of the 3 777 broiler carcasses, 97 (86 belonging to the post evisceration group and 11 to the post chilling group) were classified as dirty in terms of levels of visual faecal contamination.

The data collected were analysed in order to address project tasks; a multilevel mixed linear modelling for hierarchical data was used with the aim of investigating the effect of slaughterhouse, batch and carcass variables on *E. coli* and *Enterobacteriaceae* counts. The statistical analysis resulted in the following conclusions.

3.1 First task: collect relevant data on the variability of the counts of *E. coli* and *Enterobacteriaceae* on neck skin of broiler carcasses sampled at post evisceration as well as at post chilling

Even though the data set showed a wider dispersion of *E. coli* counts, it was possible to draw the same conclusions for both the indicator bacteria *E. coli* and *Enterobacteriaceae*.

- Bacterial loads are higher in broiler carcasses sampled at post evisceration compared to post chilling, and this difference proved to be statistically significant (based on model M1). The average values of *Enterobacteriaceae* counts were 4.33 and 4.00 $\log_{10}$ cfu/g at post evisceration and post chilling, respectively. The average values of *E. coli* counts were 4.16 and 3.76 $\log_{10}$ cfu/g at post evisceration and post chilling, respectively.

- Variability in bacteria counts data distribution is evident both among slaughterhouses and among batches within the same slaughterhouse. Specifically looking at the data aggregated per slaughterhouse, the magnitude of the reduction in bacterial loads between post evisceration and post chilling and the bacterial level recorded at post evisceration varies among the slaughterhouses (Figures 12 and Figure 8 in Appendix I and Tables 2 and 3 in Appendix H). Additionally, considering individual slaughterhouses, it appears that in almost all the cases a reduction in bacterial levels occurred between post evisceration and post chilling and that the magnitude of this reduction varied widely among batches.

- The bacterial counts were lower in broilers of the weight category 2-3 kg compared to the other two weight categories both at post chilling and at post evisceration. No significant difference was stated between the weight categories <2 kg and >3 kg (based on M1 and M3).

- The higher the counts at the post evisceration are, on average, the higher the counts on the single carcasses are at post chilling. This effect is more evident for the batches with broilers below 2 kg and between 2-3 kg compared to the batches with broilers of higher weight.

The importance of the slaughtering phases following after evisceration in reducing the bacterial loads on carcasses has been investigated and demonstrated by several authors. In particular, in studies that investigated the effect of single steps, washing and chilling were identified as the

phases that determine a decrease of bacterial loads (Gonzalez-Miret et al., 2006; Goksoy et al., 2004; Northcutt et al., 2003; Berrang and Dickens, 2000; Kemp et al., 2001; Oyarzabal et al., 2004; Vaidya et al., 2005; Cox et al., 2010; Gill et al., 2006; Gill and Badoni, 2005). In the present study, the statistical models' outputs did not allow the identification of any of the phases between evisceration and chilling as responsible for significantly affecting the bacterial counts. This could be due to the peculiar characteristics of the study itself, the specific aim of which was not to explore particular steps of the slaughter process.

3.2 Second task: collect information, such as structural and managerial data about the slaughterhouses visited, as well as specific information about the sampled batches, to explain the variability of the counts

To investigate the effect of slaughterhouse, batch and carcass variables on *E. coli* and *Enterobacteriaceae* counts on broiler carcasses, multilevel mixed linear models for hierarchical data were used.

In this context, slaughterhouse, batch and carcass identify three levels of clustered data sets. Such study design allows the investigation of whether covariates measured at each hierarchy level (level 1, carcass; level 2, batch; level 3, slaughterhouse) have an impact on bacterial counts, measured at level 1 of the data structure.

Results, in terms of statistically significant variables affecting bacterial counts, are reported below, keeping the three hierarchy levels separated.

3.2.1 Significant variables at carcass level

The visual classification of carcasses into "dirty" or "clean", the sampling point (post evisceration or post chilling) and the interaction between these two variables are significant in explaining the variability of bacterial counts on broiler carcasses (based on M1).

In particular, bacterial levels (both *E. coli* and *Enterobacteriaceae*) were higher in carcasses sampled at post evisceration compared to post chilling, and in dirty carcasses compared to clean ones. The significance of the interaction underlines that the difference related to the sampling points is independent from the contamination *status* identified through the visual inspection, whereas the effect of the visual evaluation is important solely at the evisceration point.

Considering only the *E. coli* and *Enterobacteriaceae* counts observed at post evisceration, the variable "visual evaluation of carcasses" proved to be significant. Specifically the average bacterial level detected in carcasses classified as dirty was significantly higher than for those classified as clean (based on M2).

The variable "visual evaluation of carcasses" was recognised as not statistically significant based on M3 and M4, which consider bacterial counts recorded solely at post chilling.

To conclude, the outputs of the models established that indicator bacterial loads are significantly higher in carcasses classified as dirty exclusively at the post evisceration sampling point.

3.2.2 Significant variables at batch level

The weight of the carcasses within the batch (weight category of the batch) and the interaction between batch weight category and sampling point were significant in explaining the variability of bacterial counts (both *E. coli* and *Enterobacteriaceae*) as reported previously (based on M1) (task 1).

M2, which only takes into account the bacterial counts at post evisceration, includes two scenarios in terms of variables that proved to be significant as regards *E. coli* counts; the second of these two scenarios also affected *Enterobacteriaceae* counts. As regards the first scenario, which applies only to *E. coli*, the weight category and the presence of intestinal ruptures proved to be significant. In particular, batches with broilers of weight of 2-3 kg presented lower average *E. coli* counts compared to batches below 2 kg (based on M1), and batches without intestinal ruptures had lower average *E. coli* counts compared to batches with intestinal ruptures.

The second scenario, which fitted for both bacteria, confirmed that the variable "presence of intestinal rupture" was not significant.

M3 found that the weight of carcasses and the presence of discarded animals at *post mortem* significantly affect *E. coli* and *Enterobacteriaceae* counts recorded at post chilling. In particular, batches of 2-3 kg had lower bacterial levels compared to batches of <2 kg and >3 kg, and batches without discarded animals had lower counts than those with discarded animals (the difference based on $\log_{10}$ average values was 0.68 and 0.83 for *E. coli* and *Enterobacteriaceae*, respectively).

Additionally to results obtained with M3, M4 highlights the relationship between the average level of counts (both the bacteria) at post evisceration and the bacterial level (on the single carcasses) at post chilling (details in conclusions task 1).

To conclude, carcasses belonging to the weight category 2-3 kg proved to be less contaminated with both indicator bacteria either at post evisceration and at post chilling compared to other weight categories (<2 kg and > 3 kg). This could be due to the fact that the majority of the slaughterhouses facilities are appropriate for medium sized birds, and even though adaptation to the broiler size is possible, this could lead to some problems during the slaughtering process.

This hypothesis is in agreement with Russel (2003), who stated that smaller broilers are more difficult to process because the evisceration equipment cannot automatically adjust for smaller sized carcasses; moreover, they stated that processing errors may results from improperly adjusted or worn-out evisceration equipment, variance among individual birds, or birds with low body weight. These variables are important because poultry processing is a highly automated operation, and the equipment is set to receive carcasses of a specific size.

However, on the other hand, this observation is not supported by the data collected within this study, in which problems related to the slaughtering process were not commonly identified as reasons for rejection of carcasses. Moreover, the event of intestinal ruptures was not frequent in any of the batch weight categories, and there is no evidence that the weight of the broilers was associated with processing errors at the evisceration step.

Certainly, the presence of discarded animals affects the indicator bacterial counts recorded at post chilling. According to the data collected, the reasons for rejection were almost all related to

problems during farming, such as cachexia, muscle alterations, hepatitis, abscesses and ascites, which supports the idea that farming practices influence the quality of broiler carcasses.

3.2.3 Significant variables at slaughterhouse level

At slaughterhouse level, whereas for *E. coli* no variable was significant, for the *Enterobacteriaceae* counts, the plucking method significantly affected the contamination level of broiler carcasses. In particular, the *Enterobacteriaceae* counts are lower when the plucking method used is the vertical disk compared to the combined techniques (vertical, horizontal and counter-rotating) (based on M1).

The second scenario of M2 (that is focused on carcasses sampled at the post evisceration), which fitted for both the bacteria, gave rise to the same result as regards the plucking method, but in addition to the vertical disk method, the horizontal method also produces lower bacterial compared to the combined techniques.

The M3 and M4 models did not identify any significant variable at slaughterhouse level to explain the counts of *E. coli* and *Enterobacteriaceae* on broiler carcasses at post chilling**.**

To conclude, the plucking method significantly affects the contamination level of both bacterial indicators at the post evisceration: counts are lower when the plucking method used is the vertical disk or the horizontal disk compared to the combined techniques.

Some authors support the idea that the defeathering step may contribute in increasing the bacterial loads on carcasses. Mead (2004) states that the defeathering step, which involves high-speed rotation of multiple metal discs bearing rubber fingers, may cause considerable scattering of bacteria from carcass surfaces with the risk of cross contamination. Berrang and Dickens (2000) state that the defeathering represents an opportunity for contamination because of the contact between the picker fingers and the abdomen of the carcass, which could cause the release of gut content still present in the bowel.

3.3 Third task: to compare *E. coli* and *Enterobacteriaceae* counts on the carcasses with their categorization in terms of levels of visual faecal contamination.

A total of 3 777 samples of neck skin were submitted for laboratory analysis to quantify *E. coli* and *Enterobacteriaceae*. Despite the effort to collect dirty carcasses without affecting randomization criteria, only 97 out of the 3 777 sampled carcasses were classified as dirty in terms of visual faecal contamination level. The classification of the carcasses was conducted both according to the study criteria and according to the criteria usually adopted by the inspectors of the selected slaughterhouses, and there was always complete agreement between the two.

Out of the 97 dirty carcasses, 11 were detected in batches with broilers belonging to the 2-3 kg weight category, 26 in batches with broilers < 2 kg and 60 in the category > 3 kg.

Higher mean counts of *E. coli* and *Enterobacteriaceae* were detected in carcasses classified as dirty at the post evisceration sampling point; on the other hand, in most cases, carcasses with high bacterial counts were not classified as dirty.

The probability of failure to classify as “dirty” those carcasses that are heavily contaminated with indicator bacteria was estimated, and results demonstrate that even though the inspector has greater

chance not to fail at the post evisceration inspection point, the best probability of success is less than 13% when the bacterial counts are higher than the value corresponding to the 70th percentile.

These conclusions are supported by other studies. Specifically, for data collected on artificially contaminated carcasses, it has been demonstrated that *Enterobacteriaceae* and *E. coli* counts in samples taken at different stages along the slaughter processing line are generally not influenced by the level of faecal contamination of carcasses, especially when samples are collected at the end of the slaughter processing line. Jimenez et al. (2003) compared *E. coli* and *Enterobacteriaceae* counts on naturally contaminated carcasses with and without visual faecal contamination at different steps of the slaughter processing line (after evisceration, after washing, and after chilling), and stated that after evisceration, the counts of visually contaminated carcasses were significantly higher only for *E. coli* and that after chilling (with 19 ppm of chlorinated water) no differences resulted for both the indicator bacteria. A similar investigation, conducted in seven processing plants in the US, compared the *E. coli* counts on broiler carcasses with and without visible ingesta contamination at the pre- and post-immersion chilling steps in which chlorine was applied at different concentrations. No statistically significant differences in *E. coli* loads between these two groups were detected either before or after chilling. These finding suggested the lack of direct correlation between the presence of visible faecal material and *E. coli* contamination on carcasses (Bilgili et al., 2002).

To conclude, the inspector has an extremely low probability of classifying a carcass with high indicator bacteria counts as dirty. Moreover this ability is limited to the post evisceration inspection point. This is in agreement with the Scientific Opinion of the BIOHAZ Panel on the public health hazards to be covered by inspection of meat (poultry), focusing on the very low sensitivity of visual inspection to detect faecal contamination. Therefore, measuring *Enterobacteriaceae* and/or *E. coli* on poultry carcasses is a more effective tool to detect faecal contamination at the slaughterhouse. As the results of the current study confirm that *E. coli* and *Enterobacteriaceae* have similar behaviour on broiler carcasses, either of them could be used as PHC. However the observed wider dispersion of the *E. coli* counts on the poultry carcasses could be an argument for using *Enterobacteriaceae* as PHC. On the other hand, the current official laboratory analytical procedures, specifically ISO 16649-2:2001 (E) and ISO 21528- 2:2004(E), for the enumeration of *E. coli* and *Enterobacteriaceae*, respectively, request a biochemical confirmation of the colonies before proceeding with their counts for *Enterobacteriaceae* only.

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APPENDICES

Appendix A: Slaughterhouse management and features (questionnaire)

GENERAL INFORMATION		
Slaughterhouse name	_____	
Slaughterhouse code	_____	
Slaughterhouse Region (NUTS) / Province	_____	
Broiler slaughtered/year	<100 000 100 000-499 999 500 000-999 999	1 000 000-4 999 999 5 000 000-9 999 999 >10,000 000
Slaughtered poultry (not broiler):	Species/ categories	N° animals/year
	□ laying hens	<100 000 100 000-499 999 500 000-999 999 1 000 000-4 999 999 5 000 000-9 999 999 >10,000 000
	□ breeder *Gallus gallus*	<100 000 100 000-499 999 500 000-999 999 1 000 000-4 999 999 5 000 000-9 999 999 >10,000 000
	□ turkey female	<100 000 100 000-499 999 500 000-999 999 1 000 000-4 999 999 5 000 000-9 999 999 >10,000 000
	□ turkey male	<100 000 100 000-499 999 500 000-999 999 1 000 000-4 999 999 5 000 000-9 999 999 >10,000 000
	□ guinea fowl	<100 000 100 000-499 999 500 000-999 999 1 000 000-4 999 999 5 000 000-9 999 999 >10,000 000
	□ duck	<100 000 100 000-499 999 500 000-999 999 1 000 000-4 999 999 5 000 000-9 999 999 >10,000 000
	□ goose	<100 000 100 000-499 999 500 000-999 999 1 000 000-4 999 999 5 000 000-9 999 999 >10,000 000
	□ game bird	<100 000 100 000-499 999 500 000-999 999 1 000 000-4 999 999 5 000 000-9 999 999 >10,000 000
	□ other (*specify*) _____ _____ _____	<100 000 100 000-499 999 500 000-999 999 1 000 000-4 999 999 5 000 000-9 999 999 >10,000 000
	□ NONE	
Broiler slaughtered/day (average)	_____	
Batches slaughtered/day (average)	_____	

Working hours/day (average)	_____
Working days/week (average)	_____
Slaughter line speed according to weight categories (broilers/minute)	< 2 kg _____
	Between 2 and 3 kg _____
	> 3 kg _____
Average time between stunning and beginning of chilling according to weight categories (minutes)	< 2 kg _____
	Between 2 and 3 kg _____
	> 3 kg _____
Number of operators at the slaughter line/s	_____
Work shift day (hours)	_____

TECHNICAL AND STRUCTURAL INFORMATION			
Is the broiler slaughter line in a single room?	□ Yes □ No		
Are there several slaughter lines?	□ Yes →	How many?	_____
	□ No		
Which is the stunning method?	□ Gas □ Electronarcosis □ Electronarcosis in water □ Gas+ electronarcosis □ Other (*specify*) _____		
Which is the killing method?	□ Double sided killers (cut both carotids)		

	□ Other (*specify*)_____
Which is the scalding method and related temperature?	□ Single-bath counterflow _____C° □ Multi-bath counterflow Bath 1 (temperature) _____C° Bath 2 (temperature) _____C° Bath 3 (temperature) _____C° □ Single bath without counterflow (temperature) _____C° □ Multi-bath without counterflow Bath 1 (temperature) _____C° Bath 2 (temperature) _____C° Bath 3 (temperature) _____C°
How long is the scalding phase according to weight categories? (minutes)	< 2 kg _____
	Between 2 and 3 kg _____
	> 3 kg _____
Which is the plucking method?	□ Vertical disk □ Horizontal disk □ Counter-rotating disk □ Vertical disk + Horizontal disk □ Vertical disk + Counter-rotating disk □ Horizontal disk + Counter-rotating disk □ Vertical disk + Horizontal disk + Counter-rotating disk
Is the plucking completed by hands?	□ Yes □ No
Are the carcasses submitted to any washing between plucking and evisceration?	□ Yes □ No
Which is the evisceration method?	□ Automatic through suction pump □ Automatic drawing □ Automatic through suction completed by hands □ Automatic drawing completed by hands □ Manual drawing

<table>
<tr><td>Does intestinal leakage occur during evisceration? (refer the observation to the last month)</td><td colspan="4">□ Always □ Often □ Sometime □ Never</td></tr>
<tr><td>Specify where the inspection point is located</td><td colspan="4">□ Post scalding □ Pre scalding
□ Post defeathering □ Pre defeathering
□ Post evisceration □ Pre evisceration
□ Post chilling □ Pre chilling
□ Post killing □ Pre killing
□ Post washing
□ Other (specify)_____</td></tr>
<tr><td rowspan="2">Are the carcasses submitted to any washing after evisceration?</td><td rowspan="2">□ Yes</td><td rowspan="2">Is there any washing between the evisceration and the inspection point?</td><td>□ Yes</td></tr>
<tr><td>□ No</td></tr>
<tr><td>□ No</td><td colspan="3"></td></tr>
<tr><td rowspan="3">Are the carcasses submitted to any decontamination washing after evisceration?</td><td rowspan="3">□ Yes →</td><td>Water temperature (°C)</td><td colspan="2">_____</td></tr>
<tr><td colspan="3">Type of washing (1) (more than one option is possible):

□ internal □ external □ intra-cavity</td></tr>
<tr><td colspan="3">Type of washing (2):

shower
high pressure (specify the water pressure with the unit of measure) _____</td></tr>
<tr><td>□ No</td><td colspan="3"></td></tr>
<tr><td rowspan="3">How long is the chilling phase according to weight categories? (minutes)</td><td colspan="4">< 2 kg _____</td></tr>
<tr><td colspan="4">Between 2 and 3 kg _____</td></tr>
<tr><td colspan="4">> 3 kg _____</td></tr>
<tr><td>Specify the chilling method</td><td colspan="4">□ Air □ Spray □ Water (Immersion)</td></tr>
</table>

<table>
<tr><td>Specify the chilling technique</td><td colspan="3">□ tunnel □ refrigerating room
□ single bath □ multi bath</td></tr>
<tr><td></td><td></td><td></td><td></td></tr>
<tr><td rowspan="6">Is the chilling temperature homogeneous? (°C)</td><td>□ Yes →</td><td colspan="2">Indicate temperature</td><td>_____°C</td></tr>
<tr><td rowspan="5">□ No→</td><td rowspan="3">Tunnel</td><td>Starting part</td><td>_____°C</td></tr>
<tr><td>Central part</td><td>_____°C</td></tr>
<tr><td>Final part</td><td>_____°C</td></tr>
<tr><td rowspan="3">Baths</td><td>Bath 1</td><td>_____°C</td></tr>
<tr><td>Bath 2</td><td>_____°C</td></tr>
<tr><td>Bath 3</td><td>_____°C</td></tr>
</table>

<table>
<tr><td>Is the chilling completed in the refrigerating room?</td><td colspan="3">□ Yes □ No</td></tr>
<tr><td rowspan="2">Is the slaughtering of the batches planned according to the health status of the flock, specifically focused on the positivity to foodborne pathogens?</td><td>□ Yes → (more than one option is possible)</td><td>□ Salmonella
□ Campylobacter
□ Other</td><td>□ S. Enteritidis
□ S. Typhimurium
□ Other serovars</td></tr>
<tr><td colspan="3">□ No</td></tr>
<tr><td>Which is the frequency of lines clean-up? (more than one option is possible)</td><td colspan="3">□ between slaughtering shifts
□ between operators shifts
□ at changing of species/ category
□ between breaks</td></tr>
<tr><td>Is the slaughter plant and supplies are in good maintenance conditions ?</td><td colspan="3">□ All □ Some
□ Most □ None</td></tr>
</table>

Appendix B: Batch information (questionnaire)

For the purpose of this study a batch of broilers is defined as a homogeneous group of broiler raised in the same farm and moved to the slaughterhouse with the same truck

GENERAL INFORMATION	
Slaughterhouse identification: company name	_____
Slaughterhouse identification: code	_____
Sampling date	_____
Batch identification *(traceability code)*	_____
Region/province where the farm of origin of the batch is located	_____
Code/s of the house/s of origin of the batch	_____
Production method at holding referred to the sampled batch (more than one option is possible):	□ Organic production □ Non-organic production □ Industrial production (Production of animals in confinement with high stocking density) □ Free range production (Animals have continuous daytime access to open air enclosures)
Outside temperature on the sampling day (°C)	_____
Sampling time	□ At the beginning of the working day □ Towards the end of the working day

Live animals transportation: information	
Start of catching date and time (hh:mm)	End of catching date and time (hh:mm)
_____ (_____:_____)	_____ (_____:_____)
Loading date and time (hh:mm)	Unloading date and time (hh:mm)
_____ (_____:_____)	_____ (_____:_____)
Weather conditions during transportation	□ Sun □ Cloud □ Overcast □ Mist □ Fog □ Light rain □ Heavy rain □ Clear □ Snow

BATCH INFORMATION	
Number of broilers of the sampled batch	_____
The broilers of the sampled batch represent	□ An entire flock □ Part of a flock
Number of animals dead at the arrival (at the slaughterhouse)	_____
Age of the broilers (days)	_____
Average weight of the broilers of the sampled batch :	□ < 2 kg □ 2 kg - 3 kg □ >3 kg
Surface area per crate (m^2)	_____
Number of broilers per crate on average	_____
Homogeneity of the animal weight within the batch? *(batch should be considered homogeneous if more than 80% of animals show approximately the same weight)*	□ Yes □ No

<table>
<tr><td rowspan="4">Is information about testing for foodborne pathogens available for the sampled batch?</td><td rowspan="3">□ Yes (more than one option is possible)</td><td>□ Salmonella</td><td>□ pos
□ neg</td><td>□ S. Enteritidis
□ S. Typhimurium
□ Other serovar</td></tr>
<tr><td>□ Campylobacter</td><td>□ pos
□ neg</td><td></td></tr>
<tr><td>□ Other</td><td>□ pos
□ neg</td><td></td></tr>
<tr><td colspan="3">□ No</td></tr>
<tr><td rowspan="2">Is the information concerning the duration of the feed withdrawal available?</td><td>□ Yes →</td><td colspan="3">Feed withdrawal duration (hours)
_____</td></tr>
<tr><td>□ No</td><td colspan="3"></td></tr>
<tr><td>Is there the presence of feed in the crop of more than 10% of the animals of the sampled batch?</td><td colspan="4">□ Yes □ No</td></tr>
</table>

OTHER INFORMATION	
Date and time (hh:mm) of slaughter beginning	_____ (_____:_____)
Date and time (hh:mm) of slaughter end	_____ (_____:_____)
Temperature of the holding pens (°C)	_____
Number of discarded animals per batch at post mortem	_____

Which is the prevalent reason of discard at post-mortem? (one option)	□ Lesions due to the farming management →	□ cachectic □ ascites □ muscle alterations □ emaciated birds □ septicaemia □ hepatitis □ pericarditis □ abscess □ other
	□ Lesions due to the slaughtering technique →	poor bleeding traumatic lesions breast burn or blister faecal contamination
% of intestinal ruptures *(estimation done considering 5% of the animals of the sampled batch)*	_____%	
Indicate EXTERNAL temperature of one carcass after evisceration (one sample)	_____°C	
Indicate EXTERNAL temperature of one carcass after chilling (one sample) *(before moving to the refrigerating room for the chilling finalization)*	_____°C	
Indicate INTERNAL temperature of one carcass after chilling (one sample) *(before moving to the refrigerating room for the chilling finalization)*	_____°C	

***ANTE MORTEM* EVALUATION OF VISUAL FAECAL CONTAMINATION (BATCH)**

According to the study criteria the batch is classified as:	□ Clean □ Contaminated
According to the slaughter criteria batch is classified as:	□ Clean □ Contaminated

***POST MORTEM* EVALUATION OF VISUAL FAECAL CONTAMINATION (BATCH)** *(immediately after evisceration)*

According to the study criteria the batch is classified as:	□ Clean □ Contaminated
According to the slaughter criteria batch is classified as:	□ Clean □ Contaminated

Appendix C: Carcass information

POST MORTEM EVALUATION OF VISUAL FAECAL CONTAMINATION (SAMPLED CARCASSES)

30 CARCASSES AFTER EVISCERATION AND 30 CARCASSES AFTER CHILLING

Carcass after evisceration			
N	Clean	Contaminated	Laboratory code
1	□	□	
2	□	□	
3	□	□	
4	□	□	
5	□	□	
6	□	□	
7	□	□	
8	□	□	
9	□	□	
10	□	□	
11	□	□	
12	□	□	
13	□	□	
14	□	□	
15	□	□	
16	□	□	
17	□	□	
18	□	□	
19	□	□	
20	□	□	
21	□	□	
22	□	□	
23	□	□	
24	□	□	
25	□	□	
26	□	□	
27	□	□	
28	□	□	
29	□	□	
30	□	□	

Carcass after chilling			
N	Clean	Contaminated	Laboratory code
1	□	□	
2	□	□	
3	□	□	
4	□	□	
5	□	□	
6	□	□	
7	□	□	
8	□	□	
9	□	□	
10	□	□	
11	□	□	
12	□	□	
13	□	□	
14	□	□	
15	□	□	
16	□	□	
17	□	□	
18	□	□	
19	□	□	
20	□	□	
21	□	□	
22	□	□	
23	□	□	
24	□	□	
25	□	□	
26	□	□	
27	□	□	
28	□	□	
29	□	□	
30	□	□	

Appendix D: Visual classification of broiler batches (photo gallery)

CONTAMINATED

CLEAN

Appendix E: Visual classification of broiler carcasses (photo gallery)

CONTAMINATED **CLEAN**

Appendix F: Information regarding the batches: categorical variables

Table 1: Sampling time (beginning/ending of the slaughtering day) of the sampled batches per slaughterhouse

	Slaughterhouse Identification (IDs)							
Sampling Time	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Sum**
Beginning	4	5	5	5	4	5	5	33
End	5	4	4	4	5	4	4	30
Sum	9	9	9	9	9	9	9	63

Table 2: Categorization of batches into weight categories per slaughterhouse

IDs	2-3 kg	Above 3 kg	Below 2 kg	*Sum*
1	0	9	0	9
2	0	0	9	9
3	6	0	3	9
4	1	8	0	9
5	0	4	5	9
6	7	0	2	9
7	9	0	0	9
Sum	23	21	19	63

Table 3: Distribution describing number of animals in the sampled batches in each slaughterhouse

IDs	Min.	Median	Mean	Max.
1	2,112	2,240	2,462	3,864
2	3,000	8,000	8,807	19,900
3	330	400	403.3	500
4	2,496	4,000	3,780	5,120
5	1,800	6,400	4,834	7,560
6	6,800	31,500	27,560	41,100
7	5,500	29,500	25,170	37,500

Table 4: Distribution of animals by age (dd) per slaughterhouse

IDs	Min.	Median	Mean	Max.
1	60	62	62.67	68
2	32	34	35.33	39
3	45	84	79.44	120
4	48	56	56.22	61
5	38	39	48.56	64
6	34	35	35.22	38
7	34	38	37.56	40

Table 5: Number of dead animals on arrival per slaughterhouse

IDs	Min.	Median	Mean	Max.
1	2	5	5	9
2	3	11	19	46
3	0	0	0	0
4	1	12	17	42
5	0	0	3,4	25
6	6	48	46	90
7	18	81	82	184

Table 6: N° of animals per crate (square metre) per slaughterhouse

IDs	Min.	Median	Mean	Max.
1	14.17	16.54	16.01	16.54
2	22.73	35.57	34.05	36.24
3	16	16	17.1	20
4	12.35	14.71	14.71	18.82
5	12	12	14.65	31.09
6	29.41	29.41	29.41	29.41
7	22.22	22.22	22.35	23.33

Table 7: Time spent loading, catching and slaughtering per slaughterhouse

IDs	Variables (short name)	Description of variables	N	Minimum	Maximum	Mean	Std Dev
1	min_delta_loading	Time for loading (minutes)	9	20	120	56.67	27.39
	min_delta_catching	Time for catching (minutes)	0				
	min_delta_sla	Time for slaughter (minutes)	9	90	195	128.33	43.87
2	min_delta_loading	Time for loading (minutes)	9	30	120	84.89	34.19
	min_delta_catching	Time for catching (minutes)	0				
	min_delta_sla	Time for slaughter (minutes)	9	25	120	67.33	34.48
3	min_delta_loading	Time for loading (minutes)	9	60	240	108.33	57.99
	min_delta_catching	Time for catching (minutes)	0				
	min_delta_sla	Time for slaughter (minutes)	9	55	180	93.89	38.71
4	min_delta_loading	Time for loading (minutes)	9	100	310	166.11	73.94
	min_delta_catching	Time for catching (minutes)	0				
	min_delta_sla	Time for slaughter (minutes)	9	45	80	63.33	11.73
5	min_delta_loading	Time for loading (minutes)	9	60	160	79.44	36.09
	min_delta_catching	Time for catching (minutes)	0				
	min_delta_sla	Time for slaughter (minutes)	9	110	525	245.56	145.61
6	min_delta_loading	Time for loading (minutes)	9	270	1130	486.67	306.54
	min_delta_catching	Time for catching (minutes)	9	270	1130	486.67	306.54
	min_delta_sla	Time for slaughter (minutes)	9	45	230	169.44	61.87
7	min_delta_loading	Time for loading (minutes)	9	342	723	470.44	111.25
	min_delta_catching	Time for catching (minutes)	9	59	382	246.78	124.07
	min_delta_sla	Time for slaughter (minutes)	9	40	422	174.67	121.43

Appendix G: Statistical test to compare data distribution between clean and dirty batches

In order to compare the distribution of counts of indicator bacteria between dirty and clean batches, two clean batches (out of the 61) were randomly selected and the corresponding subset of counts were compared to the counts of the two dirty batches by testing the hypothesis of equal mean using a "t" test, under normality distribution assumption.

This procedure was repeated 1000 times and results are reported in Tables 1 and 2 as regards *E. coli* and *Enterobacteriaceae,* respectively.

Table 1: "t" test applied to the *E. coli* counts distribution recorded on carcasses belonging to batches classified as dirty and as clean

Null hypothesis	Alternative hypothesis	% of significant p-value
H_0: $\mu_{Clean} = \mu_{Dirty}$	H_1: $\mu_{Clean} \neq \mu_{Dirty}$	65%
	H_1: $\mu_{Clean} > \mu_{Dirty}$	35.7%
	H_1: $\mu_{Clean} < \mu_{Dirty}$	29.3%

A significant difference between the average counts of carcasses belonging to dirty and clean batches occurred in 65% of the cases: in particular, dirty batches presented significantly lower counts compared to the clean batches in 35.7% of cases, while in 29.3% of cases, the dirty batches presented significantly higher counts compared to clean batches. Based on the observed data, there is no evidence that the counts of *E. coli* in the dirty batches are higher compared to the clean batches.

Table 2: "t" test applied to the *Enterobacteriaceae* counts distribution recorded on carcasses belonging to batches classified as dirty and as clean

Null hypothesis	Alternative hypothesis	% of significant p-value
H_0: $\mu_{Clean} = \mu_{Dirty}$	H_1: $\mu_{Clean} \neq \mu_{Dirty}$	64.9%
	H_1: $\mu_{Clean} > \mu_{Dirty}$	21.6%
	H_1: $\mu_{Clean} < \mu_{Dirty}$	43.3%

A significant difference between the average counts of carcasses belonging to dirty and clean batches occurred in the 65% of the comparisons. The dirty batches presented significantly lower counts compared to the clean batches in 21.6% of cases, while in 43.3% of cases the dirty batches presented significantly higher counts compared to clean batches. There is little evidence that the counts of *Enterobacteriaceae* in the dirty batches are higher compared to the clean batches.

Appendix H: Information regarding the carcasses

Table 1: External temperature recorded on carcasses at the post evisceration sampling point and external/internal temperature recorded on carcasses at the post chilling sampling point per slaughterhouse (S_ID)

IDs	Temperature °C	Minimum Temperature °C	Maximum Temperature °C	Mean Temperature °C	Std Dev
1	External temperature PO-EV	30.00	34.00	32.17	1.41
	External temperature PO-CH	8.00	13.00	10.11	1.76
	Internal temperature PO-CH	16.00	27.00	20.11	3.72
2	External temperature PO-EV	30.00	35.00	31.86	1.87
	External temperature PO-CH*	0.00	4.00	1.88	1.55
	Internal temperature PO-CH*	0.00	5.00	2.23	1.70
3	External temperature PO-EV	32.00	36.50	34.83	1.27
	External temperature PO-CH*	14.00	29.00	18.63	4.66
	Internal temperature PO-CH*	16.00	26.00	23.00	3.16
4	External temperature PO-EV	30.00	34.50	32.89	1.36
	External temperature PO-CH	0.00	7.00	4.11	2.48
	Internal temperature PO-CH	4.50	11.00	7.89	2.37
5	External temperature PO-EV	26.00	37.00	31.67	3.74
	External temperature PO-CH	5.00	16.50	9.72	3.65
	Internal temperature PO-CH	8.00	21.00	14.67	4.82
6	External temperature PO-EV	40.00	42.00	40.91	0.96
	External temperature PO-CH*	.	.	.	.
	Internal temperature PO-CH**	2.05	3.85	2.60	0.57
7	External temperature PO-EV	36.00	37.00	36.89	0.33
	External temperature PO-CH	0.50	1.90	1.21	0.51
	Internal temperature PO-CH	1.20	2.20	1.63	0.42

* 8 observations; ** data not reported

Table 2: *E. coli* loads (log_{10} cfu/g) recorded on clean and dirty carcasses sampled at the post evisceration and post chilling sampling points

IDs	Sampling point	Contamination *status*	N of carcasses	Minimum of log counts	Maximum of log counts	Mean of log counts	Std Dev of log counts	Coeff. of variation of log counts
1	PO-CH	Clean	267	2.724	6.944	4.302	0.68	15,81
		Dirty	3	3.23	4	3.621	0.385	10,63
	PO-EV	Clean	245	3.279	7.079	5.072	0.666	13,13
		Dirty	25	3.934	6.708	5.212	0.728	13,97
2	PO-CH	Clean	268	2	6.041	3.964	0.606	15,29
		Dirty	2	4.279	5.23	4.755	0.673	14,15
	PO-EV	Clean	250	3	6.973	4.84	0.739	15,27
		Dirty	20	3.74	6	5.035	0.671	13,33
3	PO-CH	Clean	270	1	5.919	2.946	0.913	30,99
		Dirty	0	.	.	.	.	
	PO-EV	Clean	265	1.301	6.505	3.313	0.968	29,22
		Dirty	2	2.398	3.322	2.86	0.654	22,87
4	PO-CH	Clean	268	2.114	6.954	4.302	0.886	20,60
		Dirty	2	4.982	5.279	5.131	0.21	4,09
	PO-EV	Clean	237	2	7.38	5.013	0.882	17,59
		Dirty	33	3.602	7.041	5.443	0.813	14,94
5	PO-CH	Clean	266	2	5.892	3.846	0.715	18,59
		Dirty	4	2.653	4.079	3.542	0.625	17,65
	PO-EV	Clean	264	2	6	3.766	0.823	21,85
		Dirty	6	3.544	6.748	4.765	1.169	24,53
6	PO-CH	Clean	270	2.491	5.491	3.635	0.567	15,60
		Dirty	0	.	.	.	.	
	PO-EV	Clean	270	2.531	5.462	3.753	0.538	14,34
		Dirty	0	.	.	.	.	
7	PO-CH	Clean	270	2.079	5.342	3.325	0.591	17,77
		Dirty	0	.	.	.	.	
	PO-EV	Clean	270	1.954	5.447	3.297	0.564	17,11
		Dirty	0	.	.	.	.	

Table 3: *Enterobacteriaceae* loads (log_{10} cfu/g) recorded on both clean and dirty carcasses sampled at the post evisceration and post chilling sampling points

IDs	Sampling point	Contamination *status*	N of carcasses	Minimum of log counts	Maximum of log counts	Mean of log counts	Std Dev of log counts	Coeff. of variation of log counts
1	PO-CH	Clean	267	3.146	6.991	4.567	0.613	13,42
		Dirty	3	3.806	4.114	3.973	0.156	3,93
	PO-EV	Clean	245	3.431	7.079	5.263	0.659	12,52
		Dirty	25	3.886	6.778	5.422	0.766	14,13
2	PO-CH	Clean	268	3.255	6.041	4.635	0.529	11,41
		Dirty	2	5	5.176	5.088	0.125	2,46
	PO-EV	Clean	250	3.531	6.778	5.085	0.674	13,25
		Dirty	20	3.987	5.869	5.168	0.49	9,48
3	PO-CH	Clean	270	1	5.944	3.102	0.924	29,79
		Dirty	0	.	.	.	.	
	PO-EV	Clean	265	1.477	6.38	3.468	0.981	28,29
		Dirty	2	2.447	4.176	3.312	1.223	36,93
4	PO-CH	Clean	268	2.431	7.079	4.378	0.844	19,28
		Dirty	2	5	5.041	5.021	0.029	0,58
	PO-EV	Clean	237	2.447	7.447	5.096	0.891	17,48
		Dirty	33	3.74	7.255	5.444	0.833	15,30
5	PO-CH	Clean	266	2.176	5.833	4.085	0.636	15,57
		Dirty	4	3.544	4.477	3.795	0.455	11,99
	PO-EV	Clean	264	2	5.914	3.969	0.785	19,78
		Dirty	6	3.681	6.82	4.868	1.181	24,26
6	PO-CH	Clean	270	2.591	5.613	3.791	0.561	14,80
		Dirty	0	.	.	.	.	
	PO-EV	Clean	270	2.653	5.623	3.92	0.561	14,31
		Dirty	0					
7	PO-CH	Clean	270	2.301	5.362	3.477	0.572	16,45
		Dirty	0	.	.	.	.	
	PO-EV	Clean	270	2.23	5.477	3.427	0.557	16,25
		Dirty	0	.	.	.	.	

Appendix I: *E. coli* descriptive statistical analysis

-Comparison of *E. coli* counts on the carcasses with their categorization in terms of levels of visual faecal contamination.

In Figure 1, *E. coli* counts distribution is reported, keeping carcasses classified as dirty and clean as regards their visual contamination *status* separated.

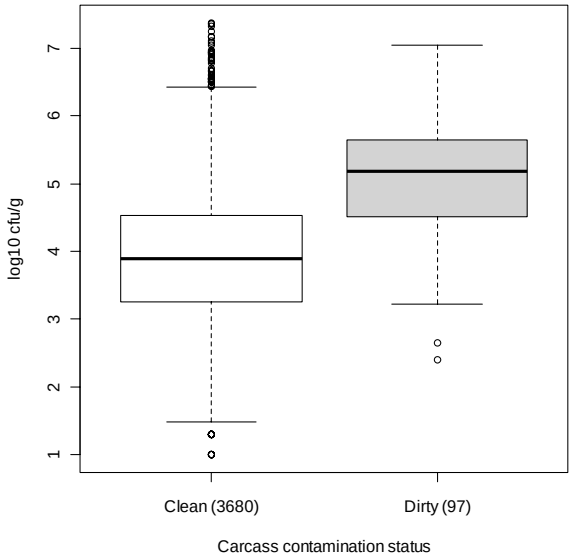

Figure 1: *E. coli* counts (log_{10} cfu/g) distribution according to the carcass visual cleanliness

In Figure 2, the previous data are expanded, keeping the two sampling points separated.

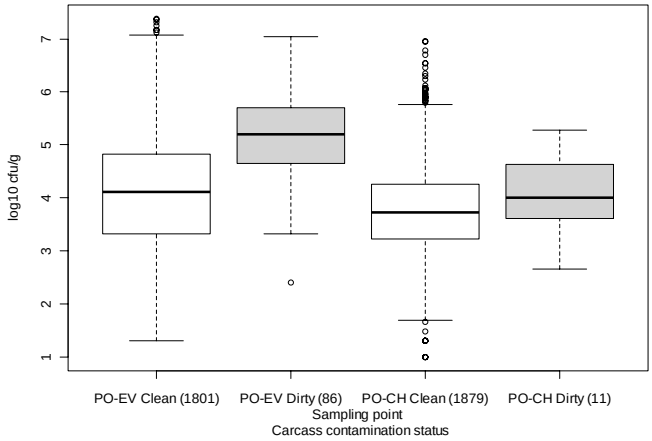

Figure 2: *E. coli* log_{10} counts (cfu/g) distribution according to the carcass contamination *status* at visual inspection toward sampling point

It is clear that higher *E. coli* counts were recorded in carcasses classified as dirty, and that a difference exists between the post evisceration and the post chilling sampling point.

Based on observed data, the probability of a carcass being classified as a "dirty carcass" both at the post evisceration and post chilling sampling points has been estimated for different *E. coli* count levels defined as "high", where "high value" corresponds to a bacterial load greater than an arbitrary cut-off value equal to the 70th percentile.

In particular the probability of failure to recognise a carcass as dirty at post evisceration is equal to 88.8%, given the *E. coli* count is higher than the 70th percentile (i.e. higher than 4.73 $\log_{10}$ cfu/g) (Figure 3).

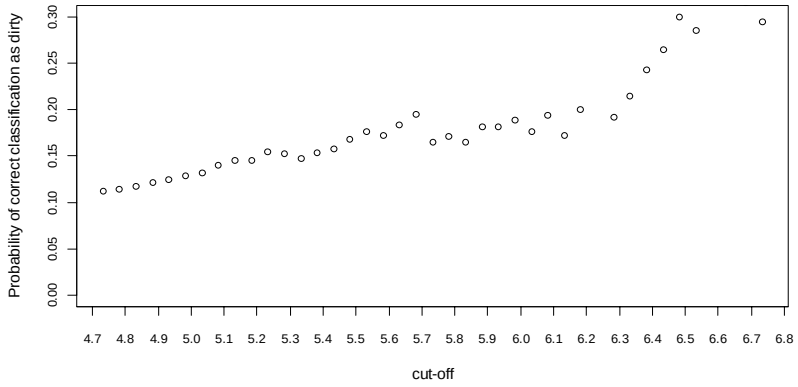

Figure 3: Probability of a carcass being classified as a "dirty carcass" for different *E. coli* cut-off values at the post evisceration sampling point

The probability of failure to recognise a carcass as dirty at post chilling is equal to 99.2%, given the *E. coli* count is higher than the 70th percentile (i.e. higher than 4.18 $\log_{10}$ cfu/g) (Figure 4).

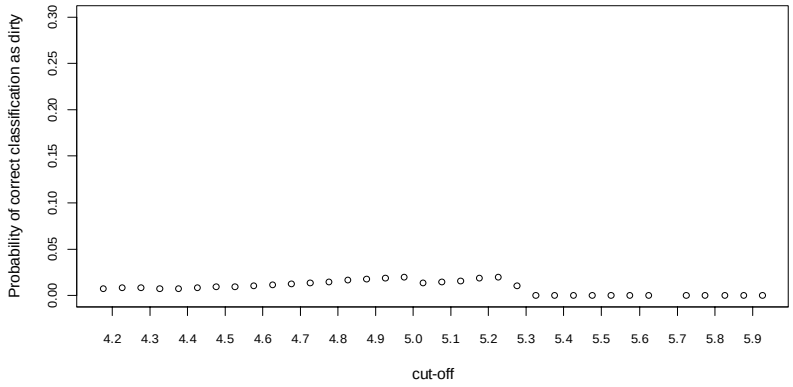

Figure 4: Probability of a carcass being classified as a "dirty carcass" for different *E. coli* cut-off values at the post chilling sampling point

Thus, the failure in classifying as dirty the carcasses that are considered heavily contaminated (values > 70th percentile) with *E. coli* according to the data collected in this study is always higher than 88%, even though it seems to be worse at post chilling.

-Data on the variability of the counts of *E. coli* on neck skin of broiler carcasses sampled at post evisceration and post chilling

In Figure 5, the *E. coli* contamination level is shown, keeping the data arising from carcasses collected at post evisceration and post chilling separated. Generally higher levels of *E. coli* were recorded at post evisceration.

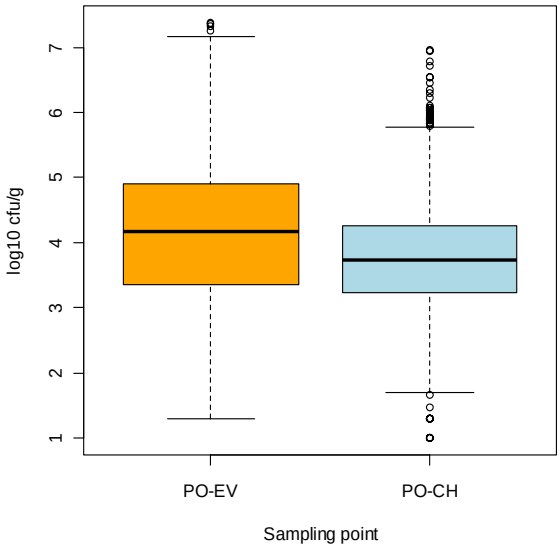

Figure 5: *E. coli* counts (log10 cfu/g) on broiler carcasses categorised by the sampling point

In Figure 6 *E. coli* counts are summarised taking into account the distribution of the batches according to the weight category of the broilers.

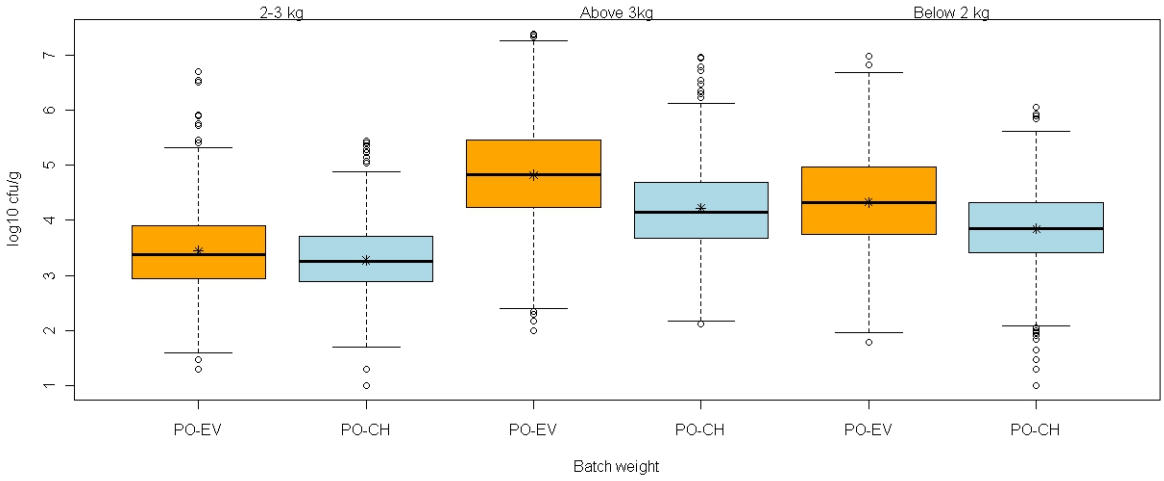

Figure 6: *E. coli* counts ($\log_{10}$ cfu/g) distribution according to broiler weight category and sampling point

Lower levels of *E. coli* were recorded in carcasses belonging to the weight category 2-3 kg.

In Figures 7 and 8, the distribution of *E. coli* levels recorded in the seven selected slaughterhouses is described; in Figure 8 counts recorded at the post evisceration and at the post chilling sampling points are separated.

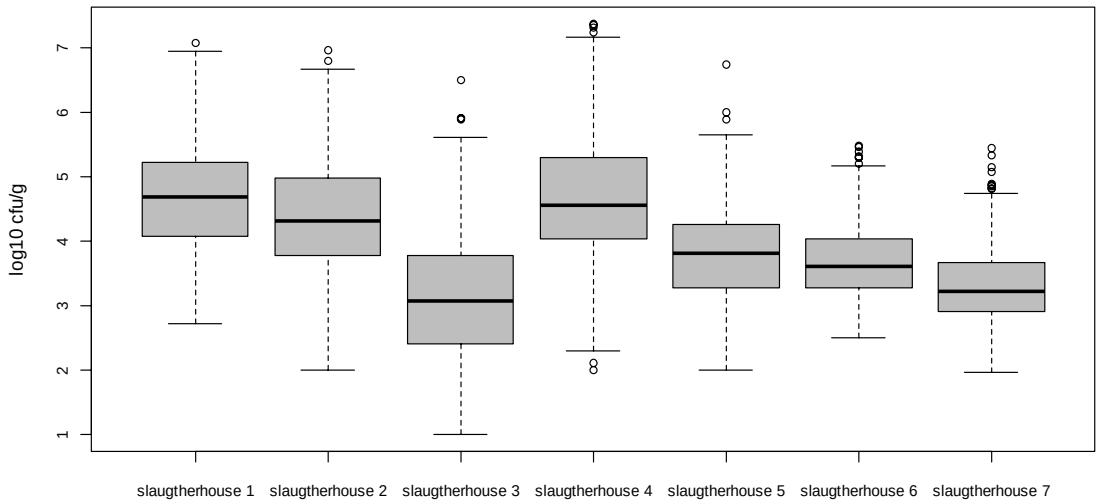

Figure 7: *E. coli* counts ($\log_{10}$ cfu/g) in the different slaughterhouses recruited for the study

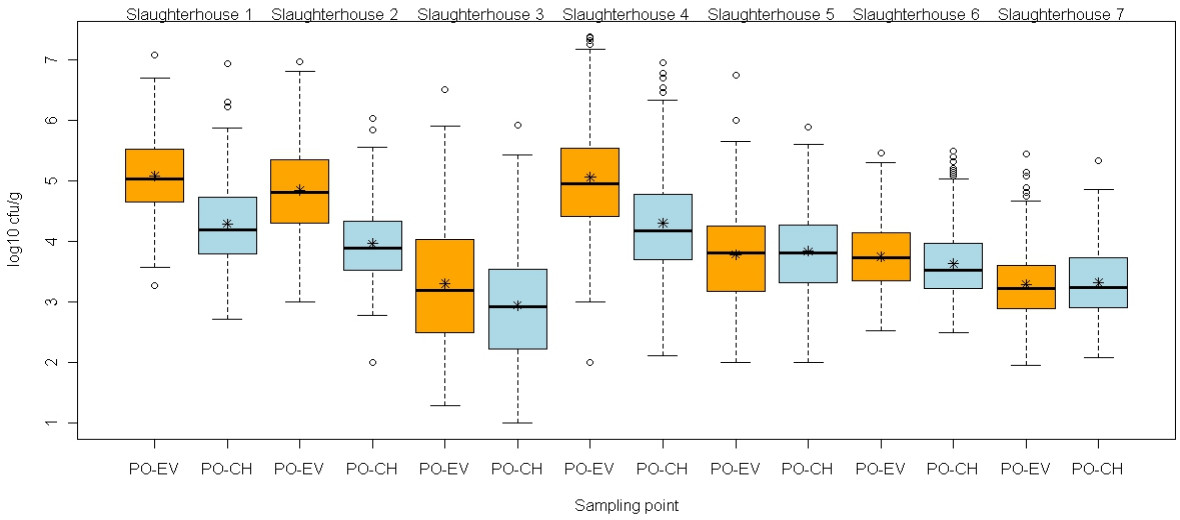

Figure 8: *E. coli* counts ($\log_{10}$ cfu/g) in the different slaughterhouses recruited for the study. Both post evisceration and post chilling sampling points are shown. * represents the mean value.

A degree of variability among the slaughterhouses is evident. The mean values are, in most cases, higher at the post eviscerations than at the post chilling sampling point.

In Figures from 9 to 18, details regarding *E. coli* counts for each batch within each slaughterhouse are shown. These data show that within the same slaughterhouse, variability exists among batches. Generally, the contamination level at post chilling is always lower to that at post evisceration, even though some exceptions are clearly visible (Figures 16, 18, 22).

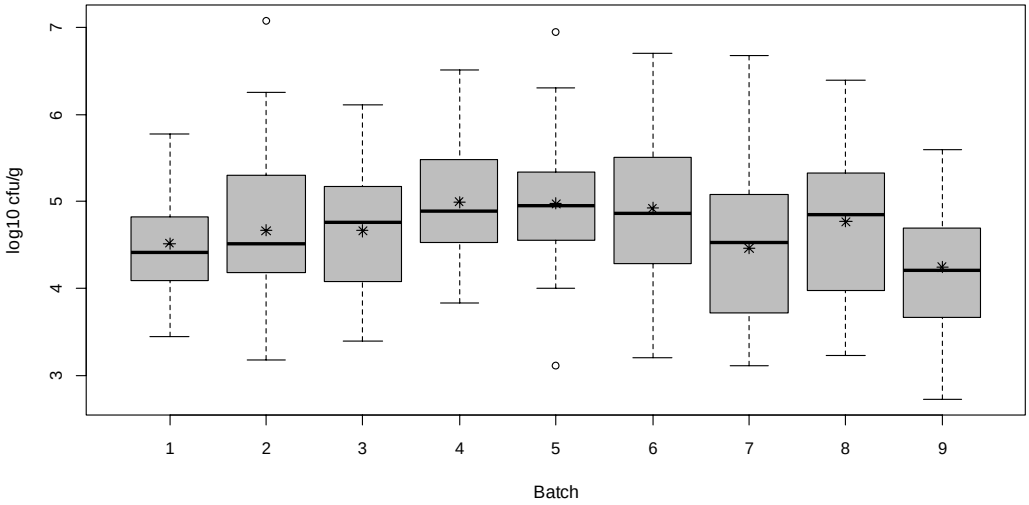

Figure 9: *E. coli* $\log_{10}$ cfu/g counts distribution per batch for slaughterhouse 1

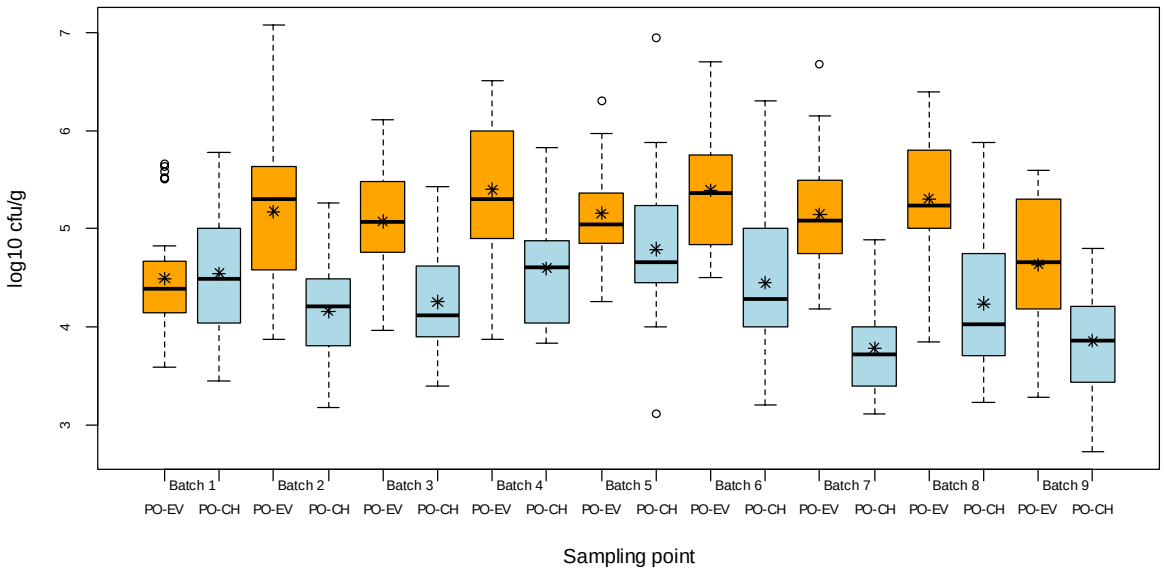

Figure 10: *E. coli* $\log_{10}$ cfu/g counts distribution per batch and sampling point for slaughterhouse 1

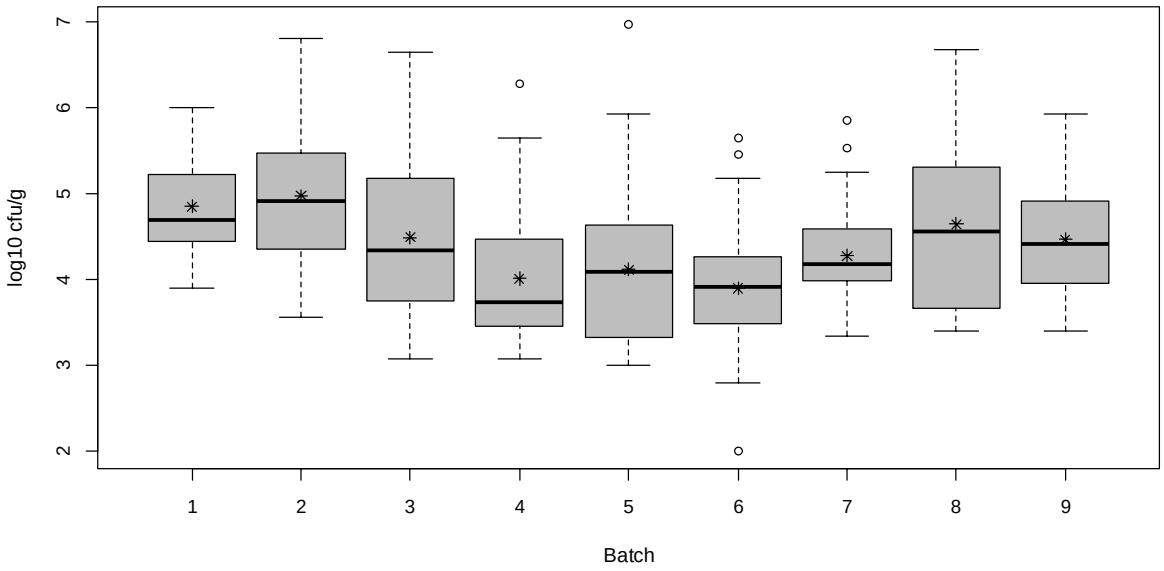

Figure 11: *E. coli* $\log_{10}$ cfu/g counts distribution per batch for slaughterhouse 2

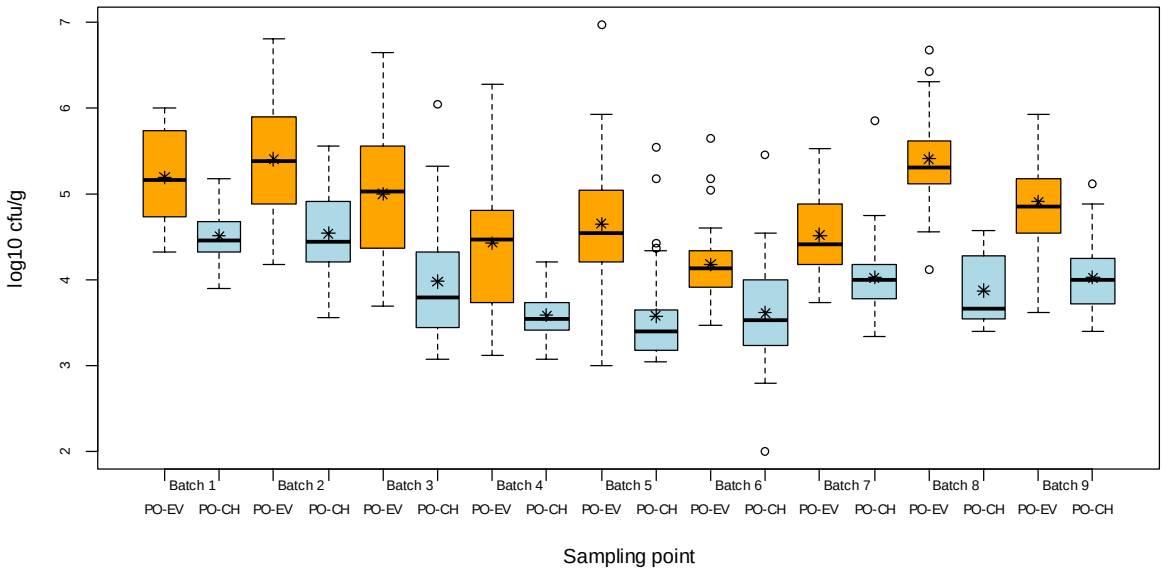

Figure 12: *E. coli* $\log_{10}$ cfu/g counts distribution per batch and sampling point for slaughterhouse 2

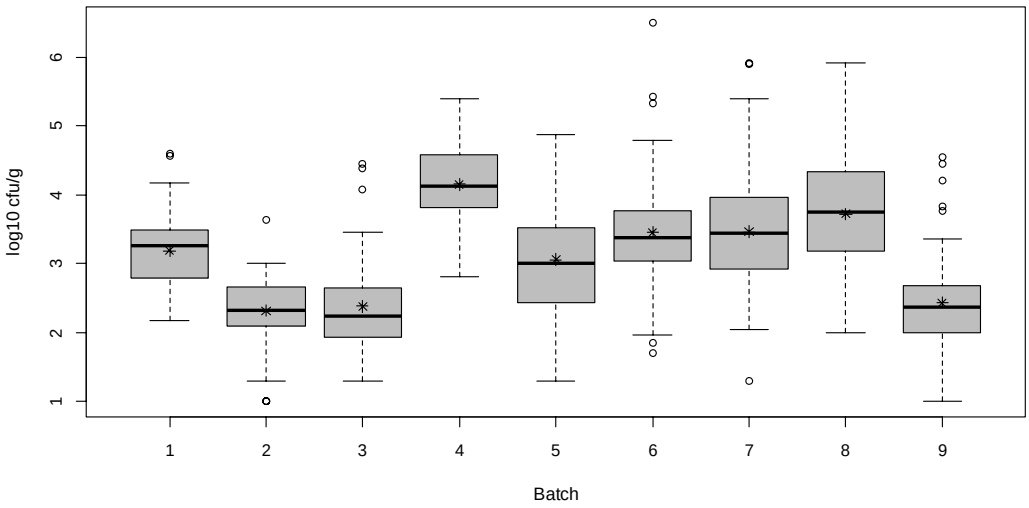

Figure 13: *E. coli* $\log_{10}$ cfu/g counts distribution per batch for slaughterhouse 3

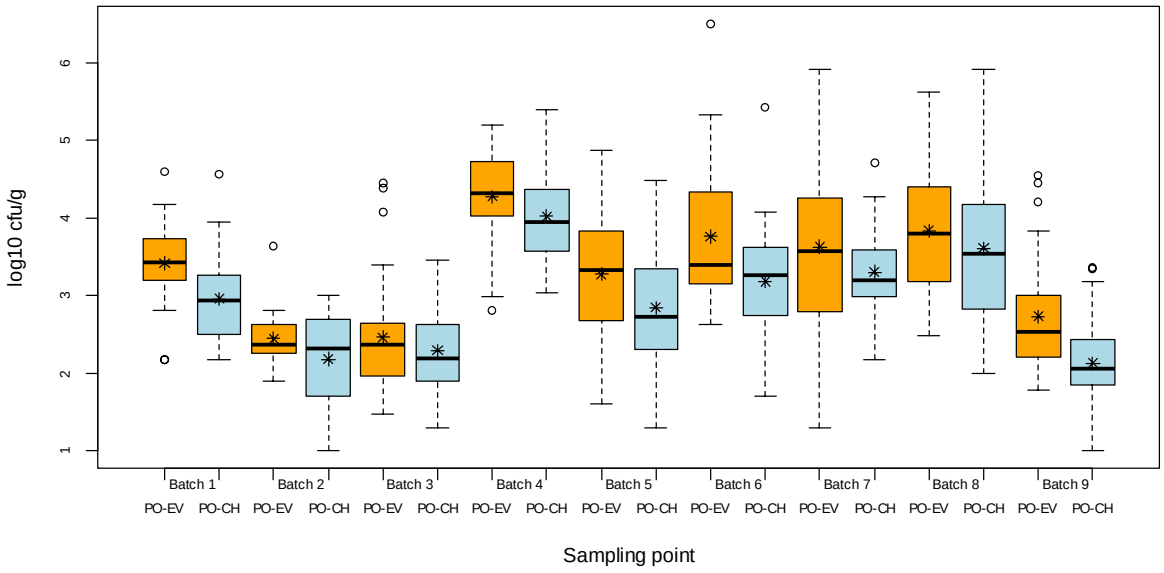

Figure 14: *E. coli* $\log_{10}$ cfu/g counts distribution per batch and sampling point for slaughterhouse 3

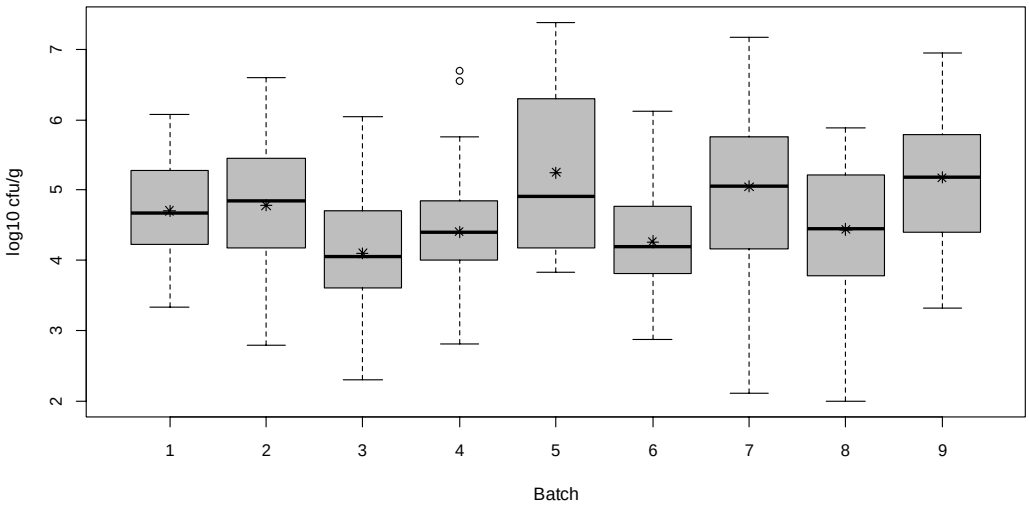

Figure 15: *E. coli* $\log_{10}$ cfu/g counts distribution per batch for slaughterhouse 4

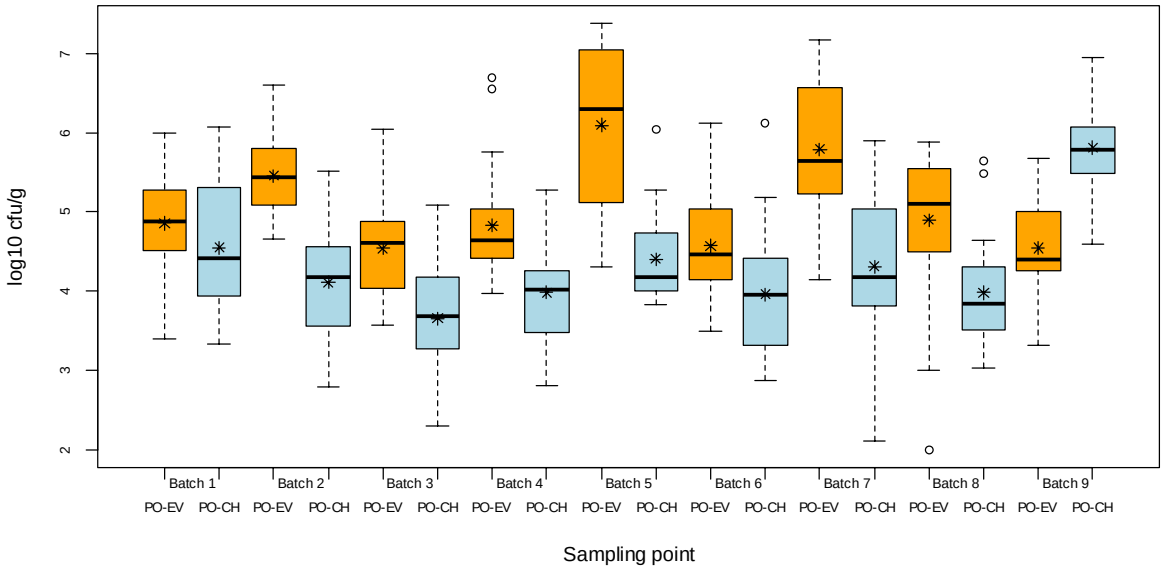

Figure 16: *E. coli* $\log_{10}$ cfu/g counts distribution per batch and sampling point for slaughterhouse 4

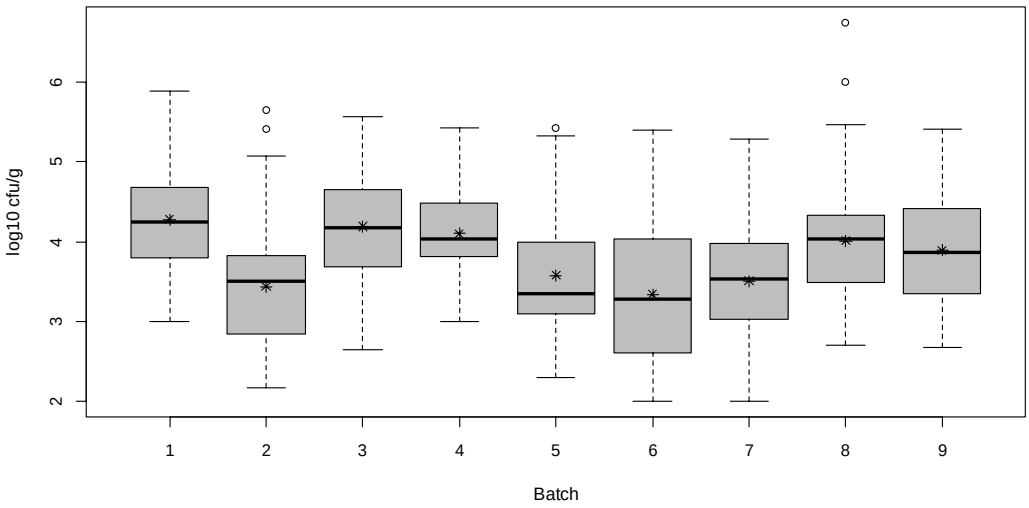

Figure 17: *E. coli* $\log_{10}$ cfu/g counts distribution per batch for slaughterhouse 5

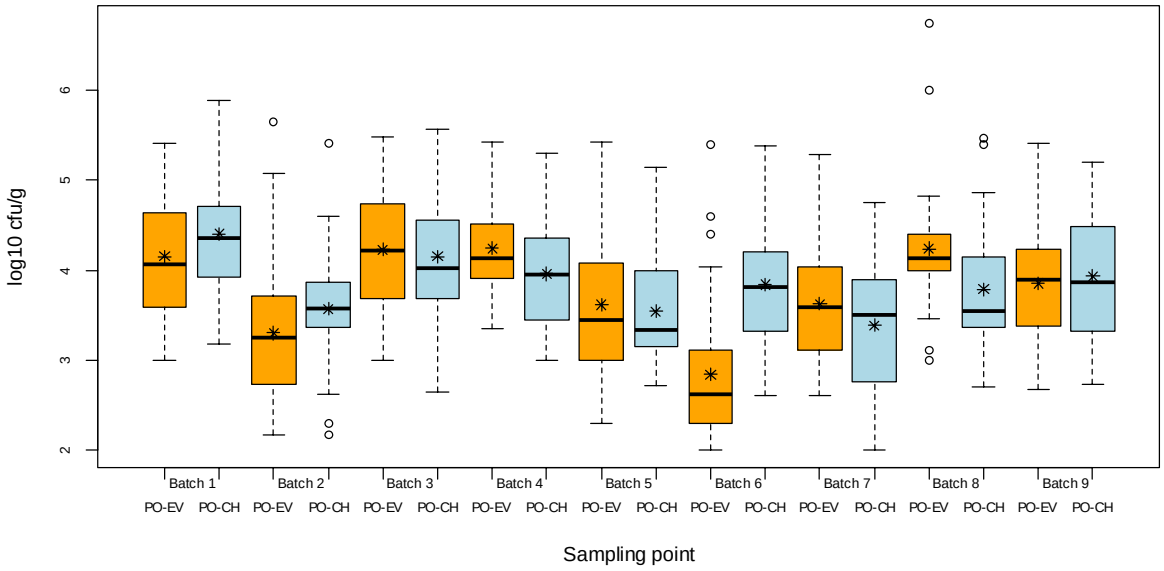

Figure 18: *E. coli* $\log_{10}$ cfu/g counts distribution per batch and sampling point for slaughterhouse 5

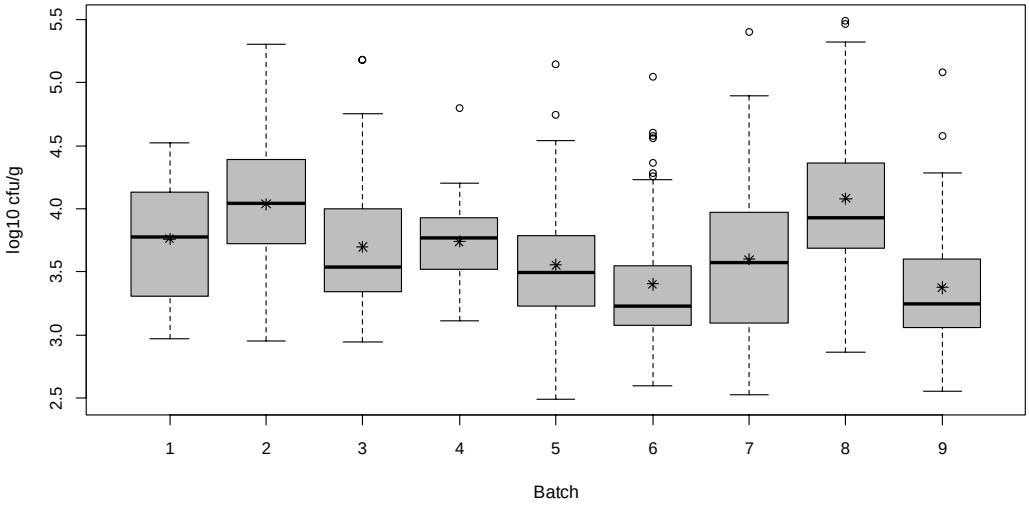

Figure 19: *E. coli* $\log_{10}$ cfu/g counts distribution per batch for slaughterhouse 6

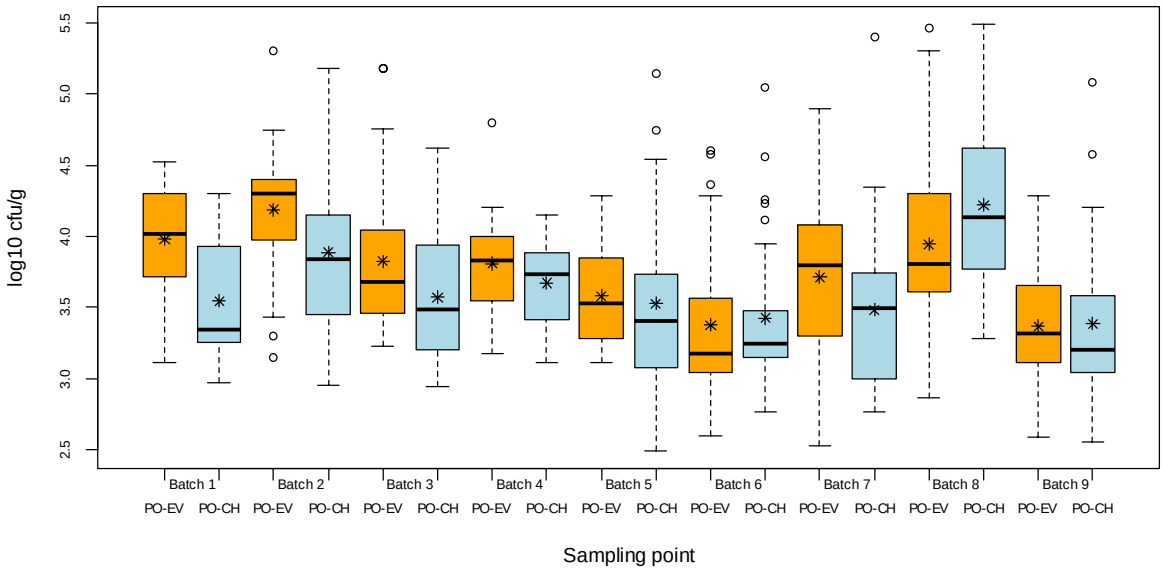

Figure 20: *E. coli* $\log_{10}$ cfu/g counts distribution per batch and sampling point for slaughterhouse 6

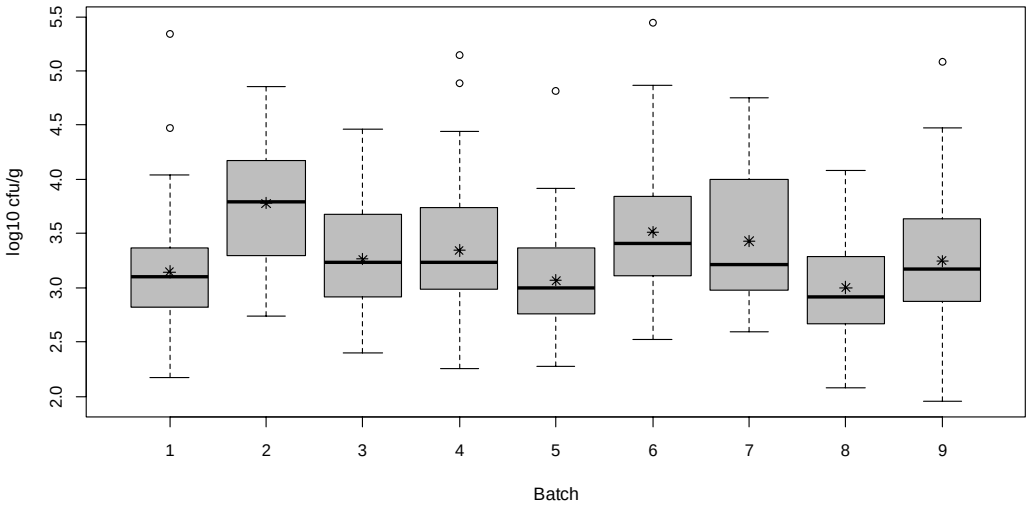

Figure 21: *E. coli* $\log_{10}$ cfu/g counts distribution per batch for slaughterhouse 7

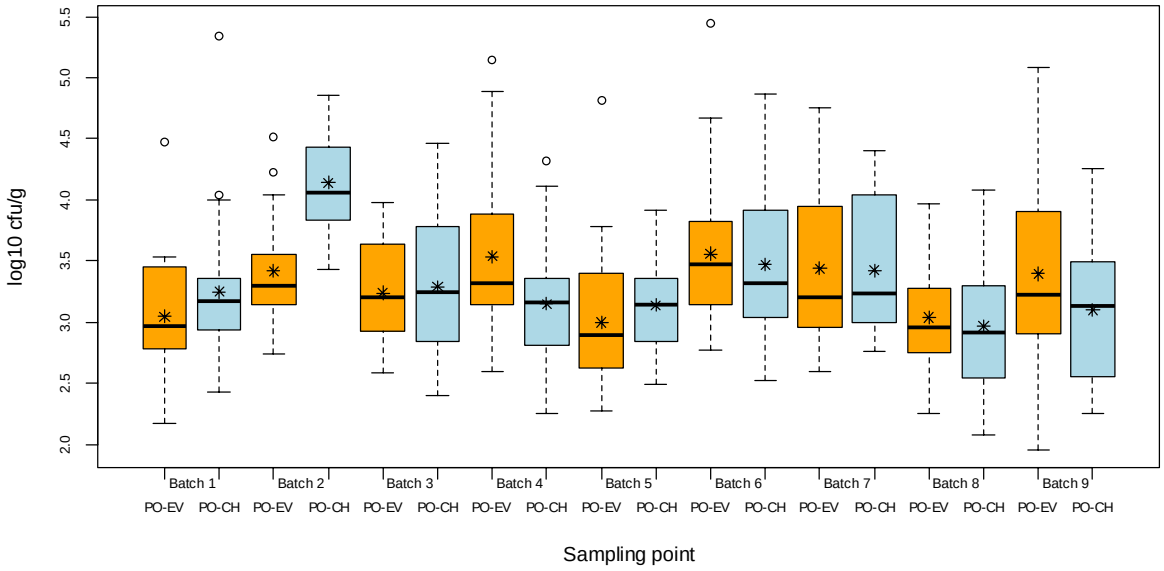

Figure 22: *E. coli* $\log_{10}$ cfu/g counts distribution per batch and sampling point for slaughterhouse 7

The possible relationship between *E. coli* counts recorded on carcasses sampled at the post evisceration and at the post chilling points were evaluated, taking into account that of necessity, different, non-corresponding carcasses were sampled at these two sampling points. Thus, the bacterial counts observed on single carcasses at post chilling were related to the average bacterial counts observed on all the sampled carcasses (i.e., representative of the batch) at post evisceration.

The graphical representation of the spline function developed for this purpose (Figure 23) shows that a linear relationship can be presumed between counts recorded at the two different sampling points.

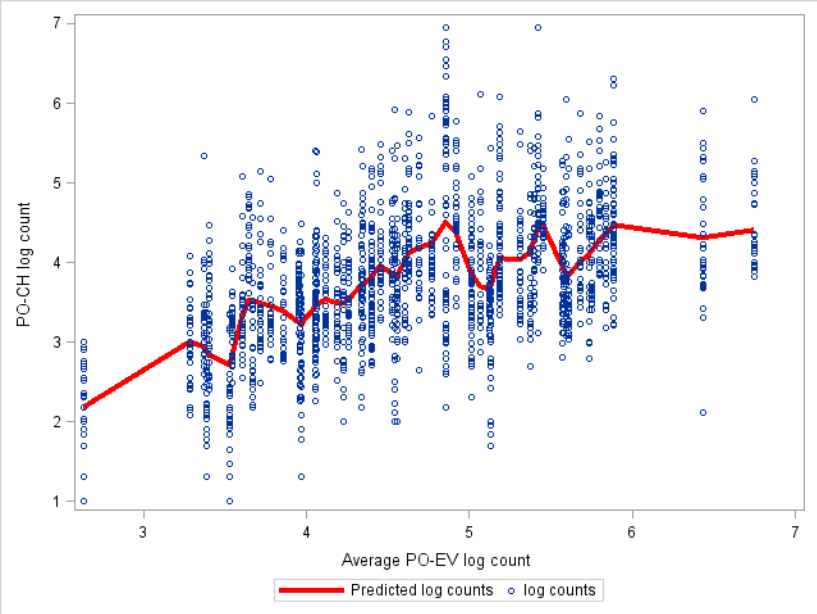

Figure 23: Relationship between *E. coli* carcass counts (log_{10} cfu/g) recorded at the post chilling sampling point and the batch average counts (log_{10} cfu/g) observed at the post evisceration sampling point

Finally in Figure 24, *E. coli* counts distribution is described keeping batches collected at the beginning of the slaughtering day and toward the end of the slaughtering day separated. These data show that the slaughter order does not affect the *E. coli* level on the carcasses.

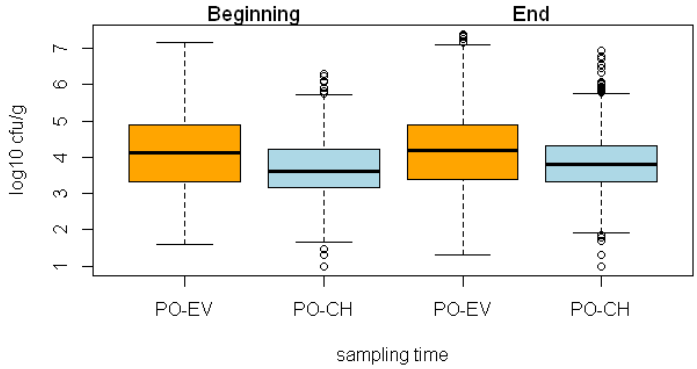

Figure 24: *E. coli* counts($\log_{10}$ cfu/g) distribution according to the time of slaughter

Appendix J: Details of models results

Table 1: M1 for *E. coli*: Significance of fixed effects

		Type 3 Tests of Fixed Effects			
	Variables (short name)	Num DF	Den DF	F Value	Pr > F
Carcass	sampMatInfo	1	3714	3.57	0.0588
	sampInfo	1	3713	37.93	<.0001
	sampMatInfo*sampInfo	1	3714	6.35	0.0118
Batch	batchWeight	2	56.6	5.41	0.0071
	batchWeight*sampInfo	2	3709	31.88	<.0001
	Fit Statistics		Covariance Parameter Estimates		
	-2 Res Log Likelihood	7857.4		Estimate	Standard Error
	AIC (smaller is better)	7863.4	$\sigma^2_{\text{int: slaugh}}$	0.2129	0.1494
	AICC (smaller is better)	7863.4	$\sigma^2_{\text{int: batch}}$	0.1309	0.02685
	BIC (smaller is better)	7863.2	σ^2	0.4424	0.01027

Table 2: M1 for *E. coli*: Differences of Least Squares Means

				Average log counts estimate		Difference estimate	Standard Error	p-value	Adjust p-value
carcass	sampMatInfo	Contaminated=N	Contaminated=Y	3.9691	4.1736	-0.2045	0.1082	0.0588	
	sampInfo	PO-CH	PO-EV	3.739	4.4037	-0.6647	0.1079	<.0001	
	sampMatInfo *sampInfo	Contaminated=N PO-CH	Contaminated=N PO-EV	3.7729	4.1654	-0.3925	0.02202	<.0001	<.0001
		Contaminated=N PO-CH	Contaminated=Y PO-CH	3.7729	3.7051	0.06778	0.2026	0.7381	0.7381
		Contaminated=N PO-CH	Contaminated=Y PO-EV	3.7729	4.642	-0.8692	0.07519	<.0001	<.0001
		Contaminated=N PO-EV	Contaminated=Y PO-CH	4.1654	3.7051	0.4603	0.2026	0.0232	0.0278
		Contaminated=N PO-EV	Contaminated=Y PO-EV	4.1654	4.642	-0.4767	0.07546	<.0001	<.0001
		Contaminated=Y PO-CH	Contaminated=Y PO-EV	3.7051	4.642	-0.937	0.2149	<.0001	<.0001
batch	batchWeight	2-3 kg	Above 3 kg	3.712	4.2482	-0.5362	0.2203	0.0185	0.0277
		2-3 kg	Below 2 kg	3.712	4.2538	-0.5418	0.1723	0.0026	0.0078
		Above 3 kg	Below 2 kg	4.2482	4.2538	-0.00561	0.199	0.9776	0.9776
	batchWeight *sampInfo	2-3 kg PO-CH	2-3 kg PO-EV	3.4963	3.9276	-0.4313	0.1135	0.0001	0.0004
		2-3 kg PO-CH	Above 3 kg PO-CH	3.4963	3.834	-0.3377	0.2218	0.134	0.1827
		2-3 kg PO-CH	Above 3 kg PO-EV	3.4963	4.6624	-1.1661	0.2459	<.0001	<.0001
		2-3 kg PO-CH	Below 2 kg PO-CH	3.4963	3.8866	-0.3903	0.1744	0.0288	0.0431
		2-3 kg PO-CH	Below 2 kg PO-EV	3.4963	4.6211	-1.1247	0.2047	<.0001	<.0001
		2-3 kg PO-EV	Above 3 kg PO-CH	3.9276	3.834	0.09363	0.2457	0.7041	0.8408
		2-3 kg PO-EV	Above 3 kg PO-EV	3.9276	4.6624	-0.7348	0.2218	0.0017	0.0028
		2-3 kg PO-EV	Below 2 kg PO-CH	3.9276	3.8866	0.04105	0.204	0.8408	0.8408
		2-3 kg PO-EV	Below 2 kg PO-EV	3.9276	4.6211	-0.6934	0.1744	0.0002	0.0005
		Above 3 kg PO-CH	Above 3 kg PO-EV	3.834	4.6624	-0.8284	0.1107	<.0001	<.0001
		Above 3 kg PO-CH	Below 2 kg PO-CH	3.834	3.8866	-0.05258	0.2009	0.7944	0.8408
		Above 3 kg PO-CH	Below 2 kg PO-EV	3.834	4.6211	-0.787	0.2268	0.0008	0.0017
		Above 3 kg PO-EV	Below 2 kg PO-CH	4.6624	3.8866	0.7758	0.2264	0.0009	0.0017
		Above 3 kg PO-EV	Below 2 kg PO-EV	4.6624	4.6211	0.04136	0.2009	0.8376	0.8408
		Below 2 kg PO-CH	Below 2 kg PO-EV	3.8866	4.6211	-0.7345	0.1126	<.0001	<.0001

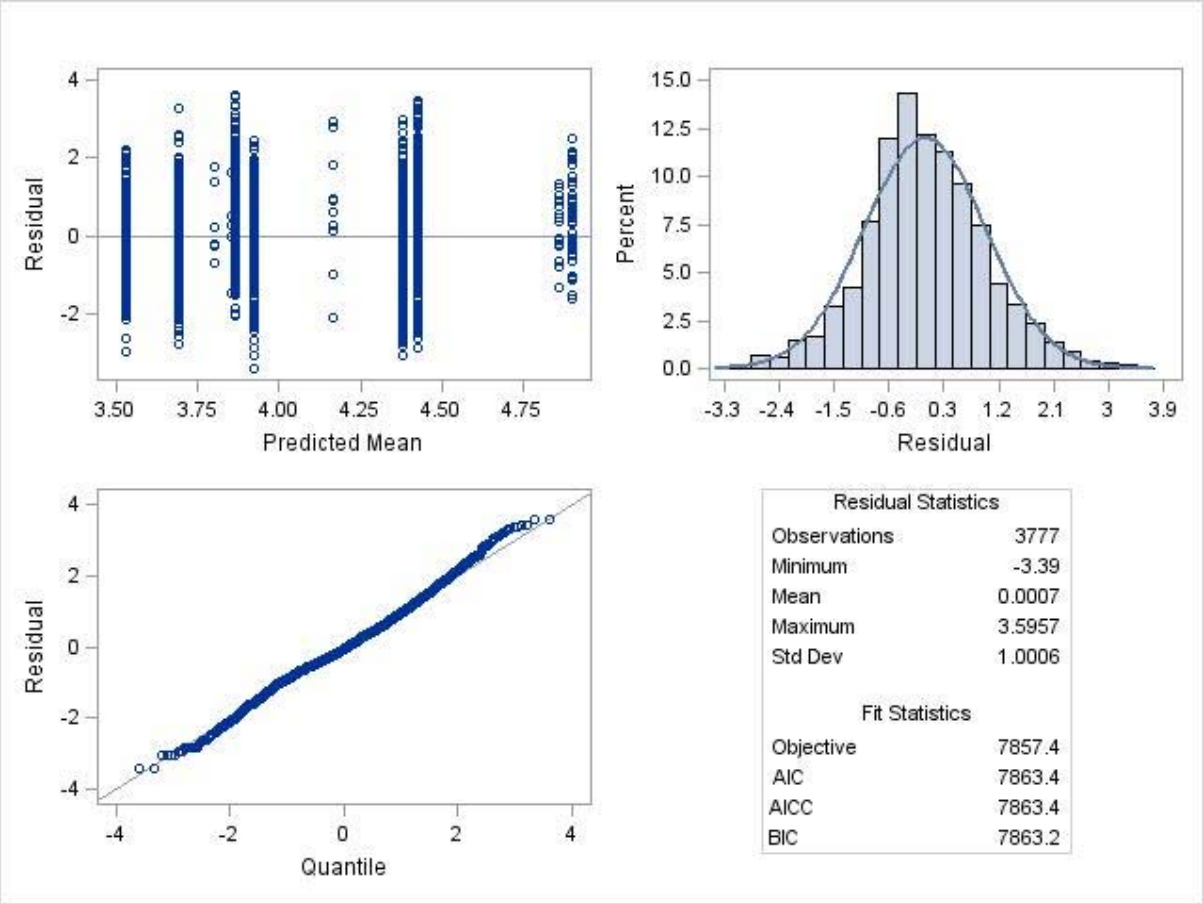

Figure 1: M1 for *E. coli*: Marginal Studentized Residuals Plots

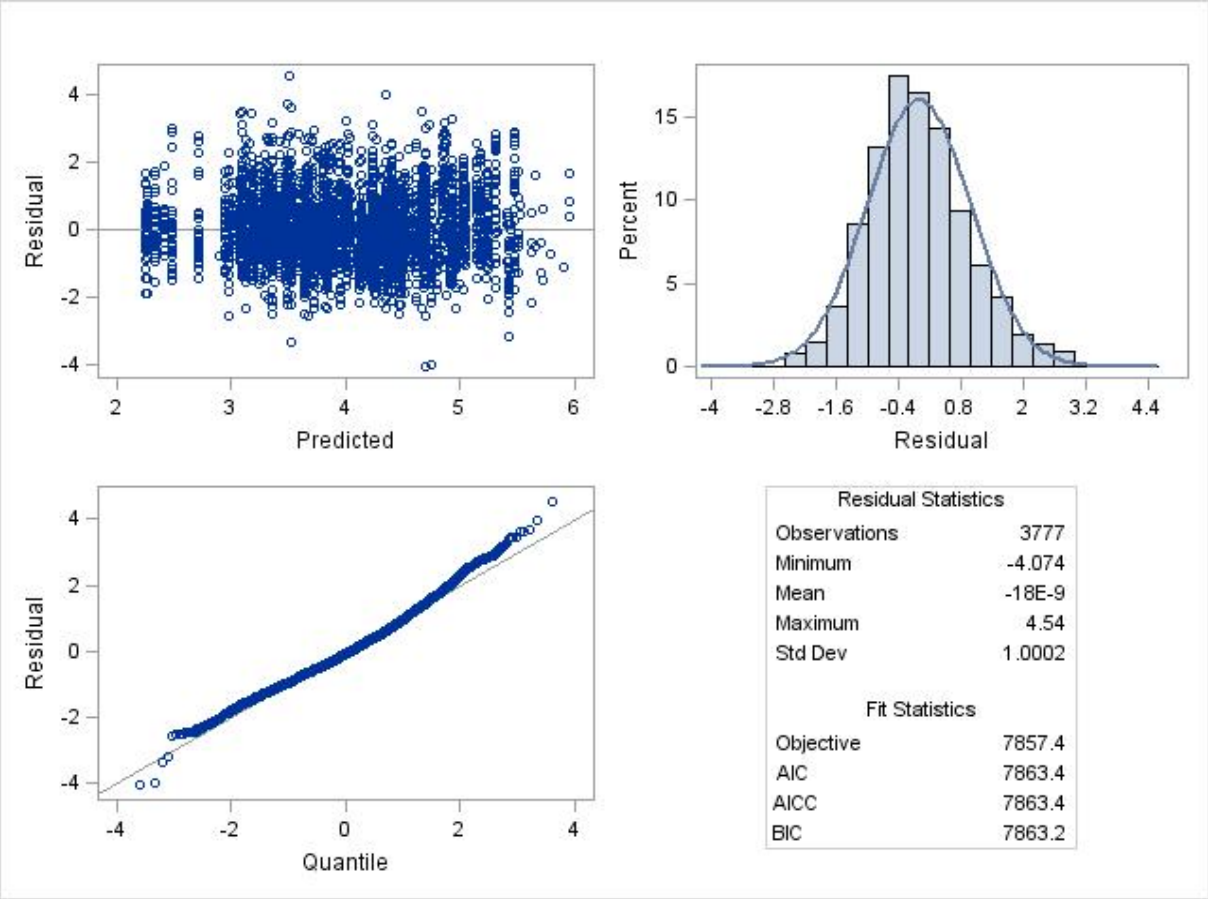

Figure 2: M1 for *E. coli*: Conditional Studentized Residuals Plots

Table 3: M1 for *Enterobacteriaceae*: Significance of fixed effects

		Type 3 Tests of Fixed Effects			
	Variables (short name)	Num DF	Den DF	F Value	Pr > F
Carcass	sampMatInfo	1	3714	1.33	0.2493
	sampInfo	1	3713	32.12	<.0001
	sampMatInfo*sampInfo	1	3713	7.01	0.0082
Batch	batchWeight	2	46,4	3.01	0.0591
	batchWeight*sampInfo	2	3709	29.41	<.0001
Slaughterhouse	defeathering	3	3.68	5.7	0.0709
	Fit Statistics		Covariance Parameter Estimates		
	-2 Res Log Likelihood	7500.4		Estimate	Standard Error
	AIC (smaller is better)	7506.4	$\sigma^2_{\text{int: slaugh}}$	0.05467	0.05765
	AICC (smaller is better)	7506.4	$\sigma^2_{\text{int: batch}}$	0.1288	0.02599
	BIC (smaller is better)	7506.2	σ^2	0.4028	0.009353

Table 4: M1 for *Enterobacteriaceae*: Differences of Least Squares Means

				Average log counts estimate		Difference estimate	Standard Error	p-value	Adjust p-value
carcass	sampMatInfo	Contaminated=N	Contaminated=Y	4.2792	4.3981	-0.1189	0.1032	0.2493	
	sampInfo	PO-CH	PO-EV	4.0468	4.6305	-0.5837	0.103	<.0001	
	sampMatInfo *sampInfo	Contaminated=N PO-CH	Contaminated=N PO-EV	4.1238	4.4346	-0.3108	0.02101	<.0001	<.0001
		Contaminated=N PO-CH	Contaminated=Y PO-CH	4.1238	3.9699	0.154	0.1934	0.4259	0.4259
		Contaminated=N PO-CH	Contaminated=Y PO-EV	4.1238	4.8264	-0.7026	0.07175	<.0001	<.0001
		Contaminated=N PO-EV	Contaminated=Y PO-CH	4.4346	3.9699	0.4647	0.1933	0.0163	0.0195
		Contaminated=N PO-EV	Contaminated=Y PO-EV	4.4346	4.8264	-0.3918	0.072	<.0001	<.0001
		Contaminated=Y PO-CH	Contaminated=Y PO-EV	3.9699	4.8264	-0.8566	0.205	<.0001	<.0001
batch	batchWeight	2-3 kg	Above 3 kg	4.0872	4.4117	-0.3244	0.2333	0.1698	0.2547
		2-3 kg	Below 2 kg	4.0872	4.5171	-0.4299	0.1754	0.0173	0.052
		Above 3 kg	Below 2 kg	4.4117	4.5171	-0.1055	0.1903	0.5829	0.5829
	batchWeight *sampInfo	2-3 kg PO-CH	2-3 kg PO-EV	3.8795	4.2949	-0.4154	0.1083	0.0001	0.0005
		2-3 kg PO-CH	Abo1ve 3 kg PO-CH	3.8795	4.0153	-0.1358	0.2346	0.5651	0.652
		2-3 kg PO-CH	Above 3 kg PO-EV	3.8795	4.808	-0.9285	0.2555	0.0005	0.0015
		2-3 kg PO-CH	Below 2 kg PO-CH	3.8795	4.2457	-0.3661	0.1772	0.0432	0.0649
		2-3 kg PO-CH	Below 2 kg PO-EV	3.8795	4.7886	-0.9091	0.2047	<.0001	0.0001
		2-3 kg PO-EV	Above 3 kg PO-CH	4.2949	4.0153	0.2796	0.2553	0.2768	0.3459
		2-3 kg PO-EV	Above 3 kg PO-EV	4.2949	4.808	-0.5131	0.2346	0.0329	0.0548
		2-3 kg PO-EV	Below 2 kg PO-CH	4.2949	4.2457	0.04925	0.204	0.8097	0.8675
		2-3 kg PO-EV	Below 2 kg PO-EV	4.2949	4.7886	-0.4937	0.1772	0.0072	0.0153
		Above 3 kg PO-CH	Above 3 kg PO-EV	4.0153	4.808	-0.7927	0.1056	<.0001	<.0001
		Above 3 kg PO-CH	Below 2 kg PO-CH	4.0153	4.2457	-0.2303	0.1921	0.238	0.3246
		Above 3 kg PO-CH	Below 2 kg PO-EV	4.0153	4.7886	-0.7733	0.2168	0.0007	0.0018
		Above 3 kg PO-EV	Below 2 kg PO-CH	4.808	4.2457	0.5624	0.2164	0.0117	0.022
		Above 3 kg PO-EV	Below 2 kg PO-EV	4.808	4.7886	0.01941	0.1921	0.92	0.92
		Below 2 kg PO-CH	Below 2 kg PO-EV	4.2457	4.7886	-0.5429	0.1074	<.0001	<.0001

				Average log counts estimate		Difference estimate	Standard Error	p-value	Adjust p-value
slaughter	defeathering	HD	VD	3.9593	3.7618	0.1975	0.3406	0.59	0.7011
		HD	VD and CD	3.9593	4.8878	-0.9285	0.3881	0.0887	0.1426
		HD	VD and CD and HD	3.9593	4.7459	-0.7866	0.3236	0.0952	0.1426
		VD	VD and CD	3.7618	4.8878	-1.126	0.3734	0.0242	0.0726
		VD	VD and CD and HD	3.7618	4.7459	-0.9841	0.2807	0.0161	0.0726
		VD and CD	VD and CD and HD	4.8878	4.7459	0.1419	0.3398	0.7012	0.7011

HD = horizontal disk; VD= vertical disk; CD= counter-rotating disk

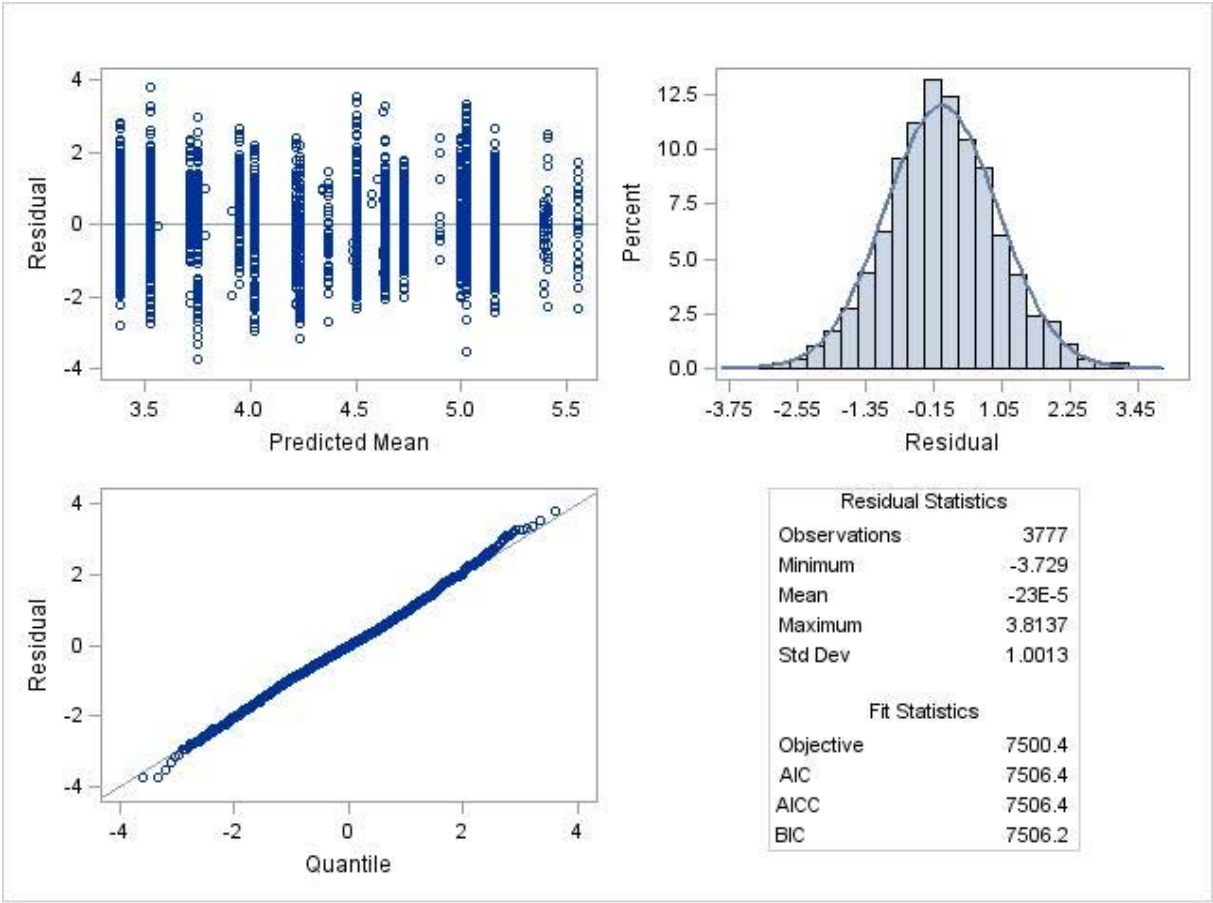

Figure 3: M1 for *Enterobacteriaceae*: Marginal Studentized Residuals Plots

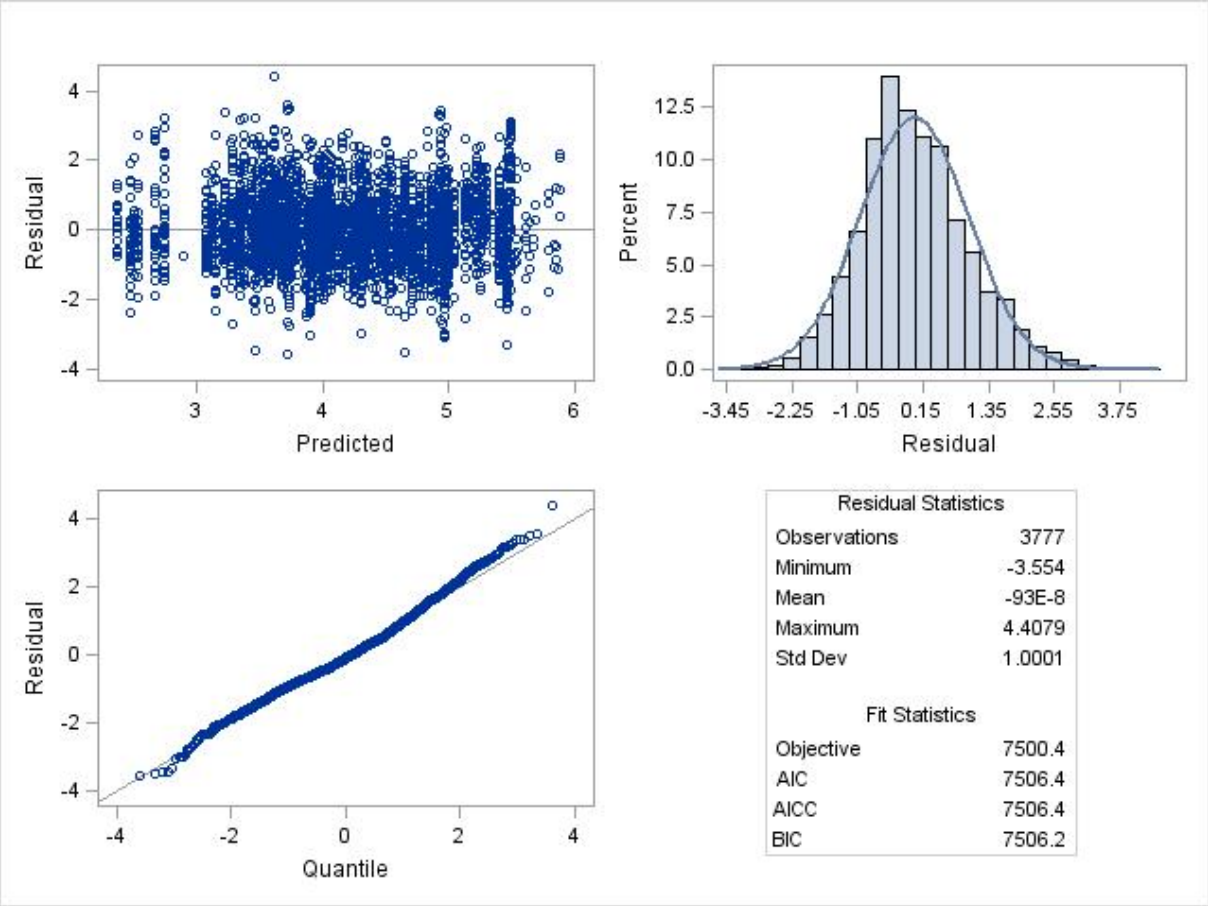

Figure 4: M1 for *Enterobacteriaceae*: Conditional Studentized Residuals Plots

Table 5: M2 for *E. coli*: Significance of fixed effects

		Type 3 Tests of Fixed Effects			
	Variables (short name)	Num DF	Den DF	F Value	Pr > F
Carcass	sampMatInfo	1	1833	16.87	<.0001
	sampInfo	°	°	°	°
	sampMatInfo*sampInfo	°	°	°	°
Batch	batchWeight	2	58.5	3.25	0.0459
	PctIntestRupture_01	1	58.7	2.86	0.0963
	Fit Statistics		Covariance Parameter Estimates		
	-2 Res Log Likelihood	3831.4		Estimate	St. Error
	AIC (smaller is better)	3837.4	$\sigma^2_{\text{int: slaugh}}$	0.4978	0.3366
	AICC (smaller is better)	3837.5	$\sigma^2_{\text{int: batch}}$	0.1701	0.03598
	BIC (smaller is better)	3837.3	σ^2	0.4032	0.01335

° : Variables that cannot be included in the model

Table 6: M2 for *E. coli*: Differences of Least Squares Means

				Average log counts estimate		Difference estimate	Standard Error	p-value	Adjust p-value
sample	sampMatInfo	Contaminate=N	Contaminated=Y	4.1749	4.476	-0.3011	0.0733	<.0001	
batch	batchWeight	2-3 kg	Above 3 kg	4.0361	4.3874	-0.3513	0.2692	0.1971	0.2957
		2-3 kg	Below 2 kg	4.0361	4.5529	-0.5169	0.2028	0.0135	0.0404
		Above 3 kg	Below 2 kg	4.3874	4.5529	-0.1656	0.2411	0.4949	0.4949
	PctIntest Rupture_01	No	Si	4.1819	4.469	-0.2871	0.1699	0.0963	

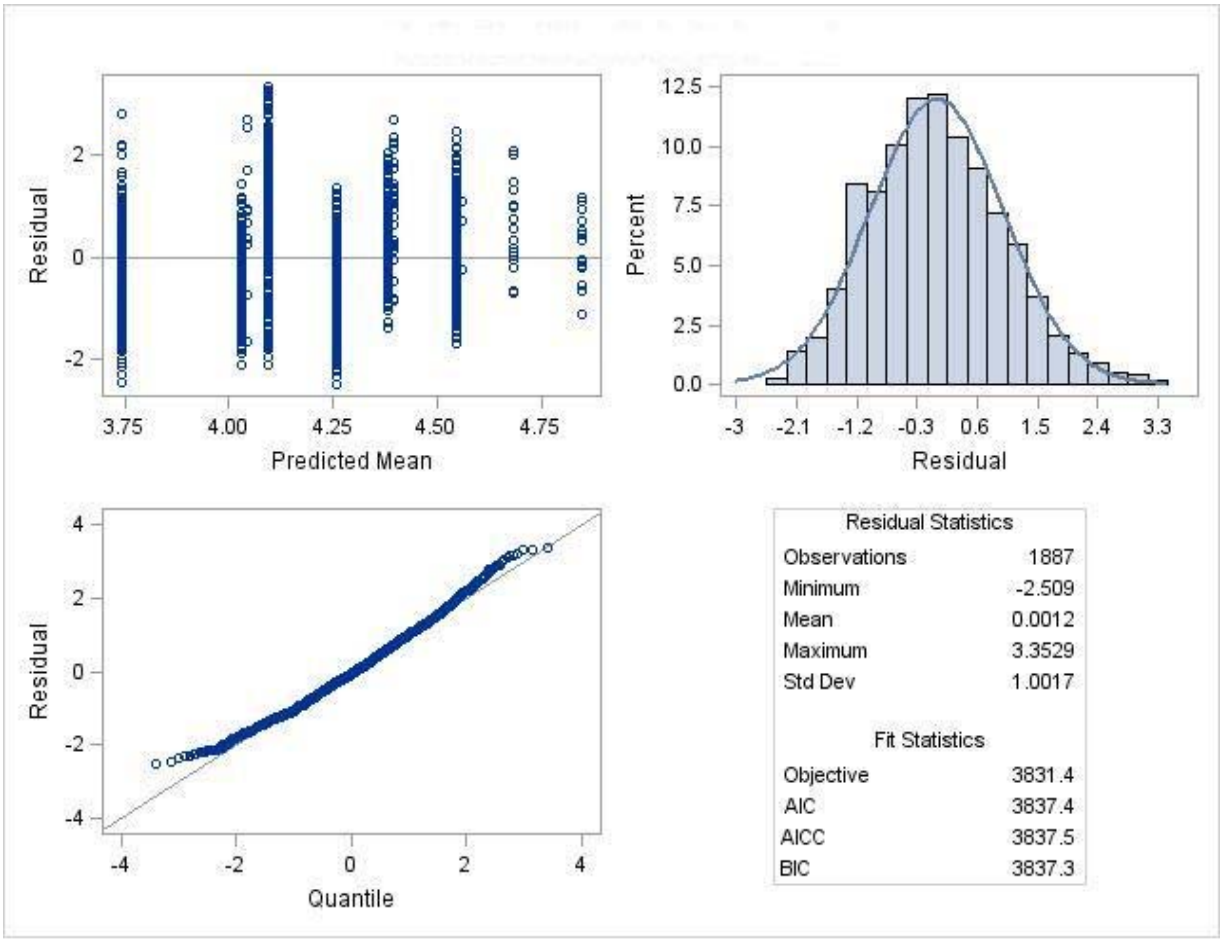

Figure 5: M2 for *E. coli*: Marginal Studentized Residuals Plots

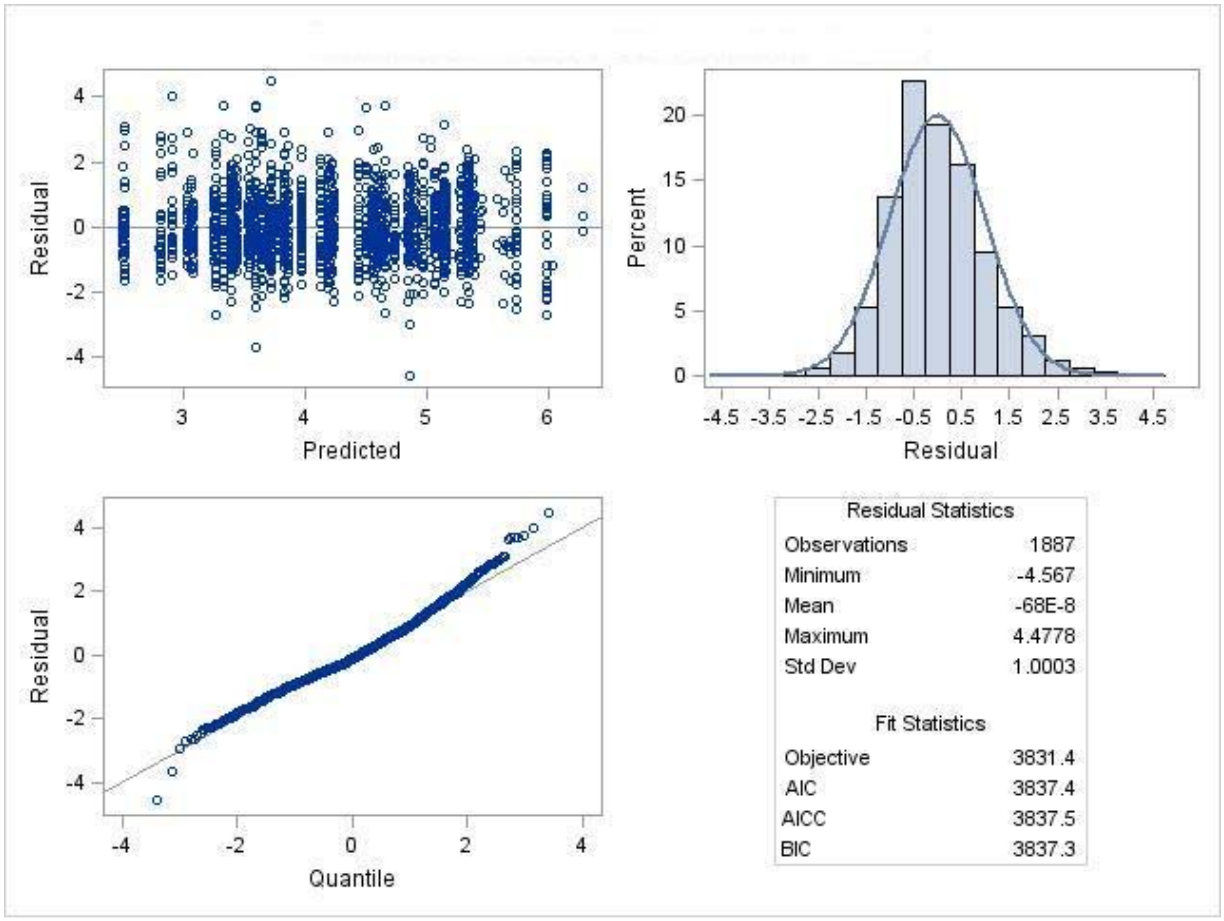

Figure 6: M2 for *E. coli*: Conditional Studentized Residuals Plots

Table 7: M2 for *E. coli* * : Significance of fixed effects

		Type 3 Tests of Fixed Effects			
	Variables (short name)	Num DF	Den DF	F Value	Pr > F
Carcass	sampMatInfo	1	1833	16.78	<.0001
	sampInfo	°	°	°	°
	sampMatInfo*sampInfo	°	°	°	°
Batch	batchWeight	2	37.4	1.99	0.1516
Slaughterhouse	defeathering	3	3.22	9.24	0.0441
	Fit Statistics		Covariance Parameter Estimates		
	-2 Res Log Likelihood	3820.3		Estimate	St. Error
	AIC (smaller is better)	3826.3	$\sigma^2_{\text{int: slaugh}}$	0.04464	0.05939
	AICC (smaller is better)	3826.3	$\sigma^2_{\text{int: batch}}$	0.1781	0.03684
	BIC (smaller is better)	3826.2	σ^2	0.4031	0.01335

° : Variables that cannot be included in the model

Table 8: M2 for *E. coli* *: Differences of Least Squares Means

				Average log counts estimate		Difference estimate	Standard Error	p-value	Adjust p-value
sample	sampMatInfo	Contaminated=N	Contaminated=Y	4.2687	4.569	-0.3003	0.07331	<.0001	
batch	batchWeight	2-3 kg	Above 3 kg	4.1698	4.5059	-0.3361	0.272	0.2221	0.3331
		2-3 kg	Below 2 kg	4.1698	4.581	-0.4112	0.2064	0.0511	0.1534
		Above 3 kg	Below 2 kg	4.5059	4.581	-0.07507	0.2151	0.7302	0.7302
Slaughterhouse	defeathering	HD	VD	3.8031	3.777	0.02601	0.3463	0.9434	0.9434
		HD	VD and CD	3.8031	5.1207	-1.3176	0.3823	0.044	0.0691
		HD	VD and CD and HD	3.8031	4.9748	-1.1717	0.3147	0.0461	0.0691
		VD	VD and CD	3.777	5.1207	-1.3436	0.3878	0.0136	0.0408
		VD	VD and CD and HD	3.777	4.9748	-1.1978	0.2893	0.0076	0.0408
		VD and CD	VD and CD and HD	5.1207	4.9748	0.1459	0.3359	0.6935	0.8322

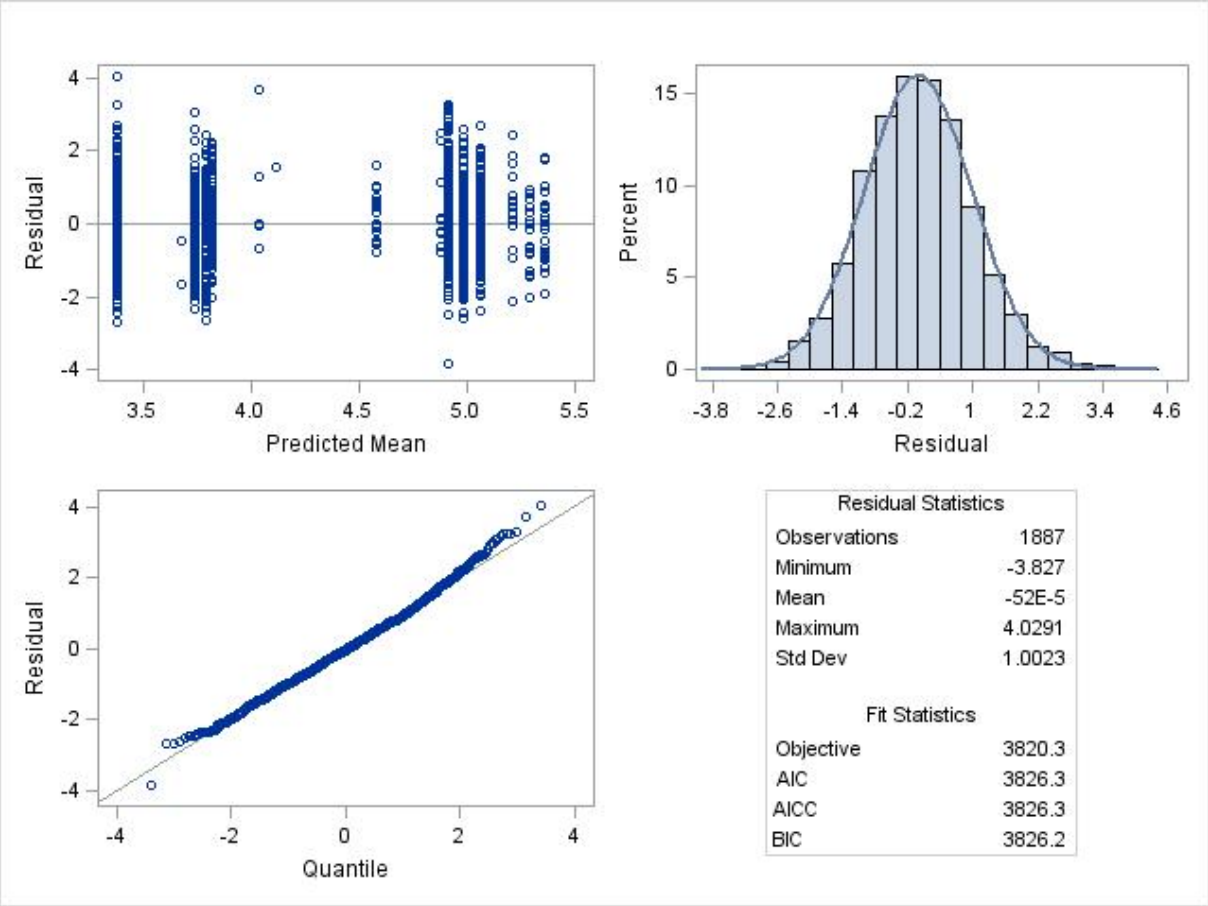

Figure 7: M2 for *E. coli* *: Marginal Studentized Residuals Plots

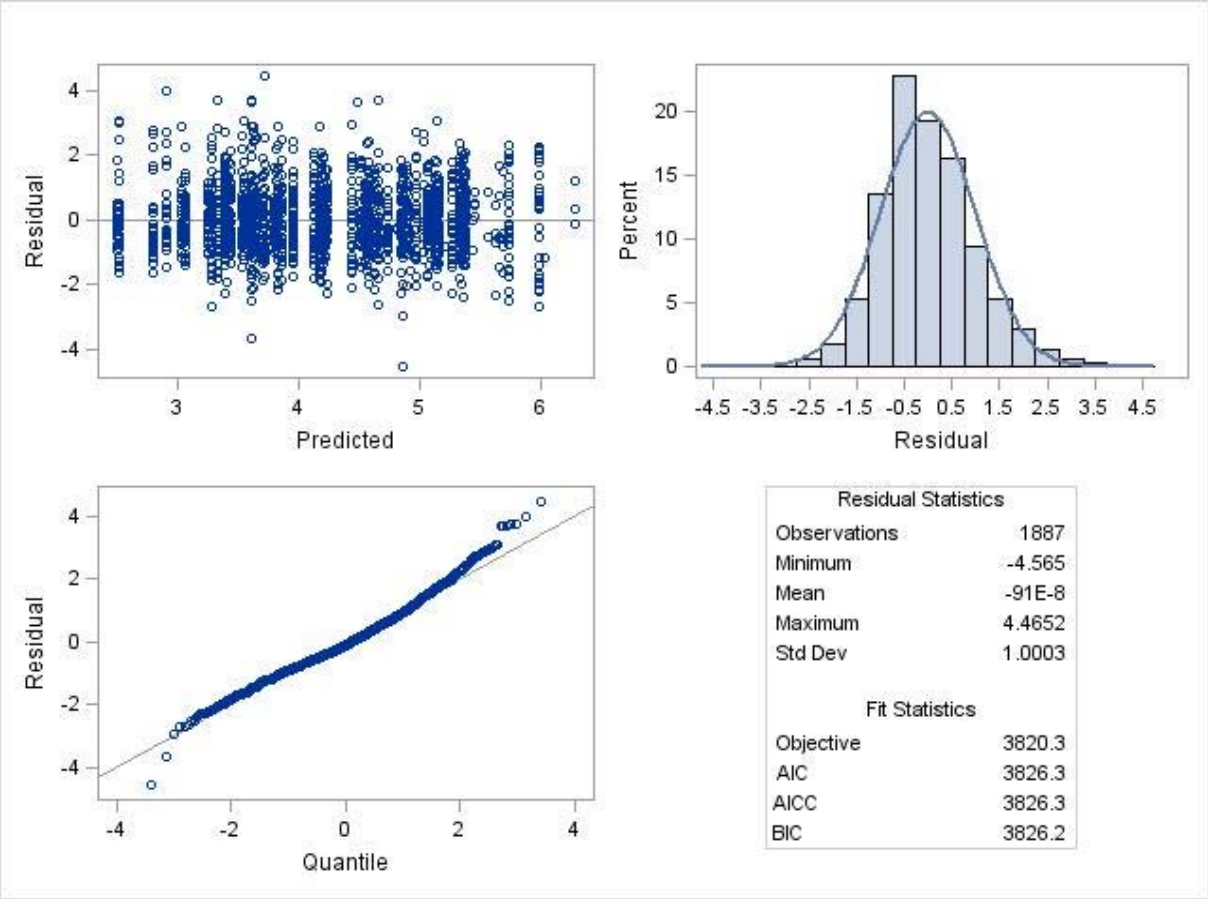

Figure 8: M2 for *E. coli* *: Conditional Studentized Residuals Plots

Table 9: M2 for *Enterobacteriaceae*: Significance of fixed effects

		Type 3 Tests of Fixed Effects			
	Variables (short name)	Num DF	Den DF	F Value	Pr > F
Carcass	sampMatInfo	1	1832	11,7	0.0006
	sampInfo	°	°	°	°
	sampMatInfo*sampInfo	°	°	°	°
Batch	batchWeight	2	35.2	2.06	0.1428
Slaughterhouse	defeathering	3	3.66	9.92	0.0305

Fit Statistics		Covariance Parameter Estimates		
-2 Res Log Likelihood	3673.3		Estimate	St. Error
AIC (smaller is better)	3679.3	$\sigma^2_{\text{int: slaugh}}$	0.03531	0.05051
AICC (smaller is better)	3679.3	$\sigma^2_{\text{int: batch}}$	0.1972	0.04018
BIC (smaller is better)	3679.1	σ^2	0.371	0.01229

° : Variables that cannot be included in the model

Table 10: M2 for *Enterobacteriaceae* : Differences of Least Squares Means

				Average log counts estimate		Difference estimate	Standard Error	p-value	Adjust p-value
sample	sampMatInfo	Contaminated=N	Contaminated=Y	4.4477	4.6882	-0.2406	0.07034	0.0006	
batch	batchWeight	2-3 kg	Above 3 kg	4.3124	4.6452	-0.3328	0.281	0.2419	0.3629
		2-3 kg	Below 2 kg	4.3124	4.7462	-0.4338	0.2144	0.0478	0.1435
		Above 3 kg	Below 2 kg	4.6452	4.7462	-0.101	0.2179	0.6479	0.6479
Slaughterhouse	defeathering	HD	VD	3.9704	3.9012	0.06914	0.3358	0.8445	0.8445
		HD	VD and CD	3.9704	5.298	-1.3277	0.3631	0.0324	0.0564
		HD	VD and CD and HD	3.9704	5.1022	-1.1318	0.2968	0.0376	0.0564
		VD	VD and CD	3.9012	5.298	-1.3968	0.38	0.0078	0.0235
		VD	VD and CD and HD	3.9012	5.1022	-1.201	0.2827	0.0043	0.0235
		VD and CD	VD and CD and HD	5.298	5.1022	0.1958	0.3197	0.5798	0.6958

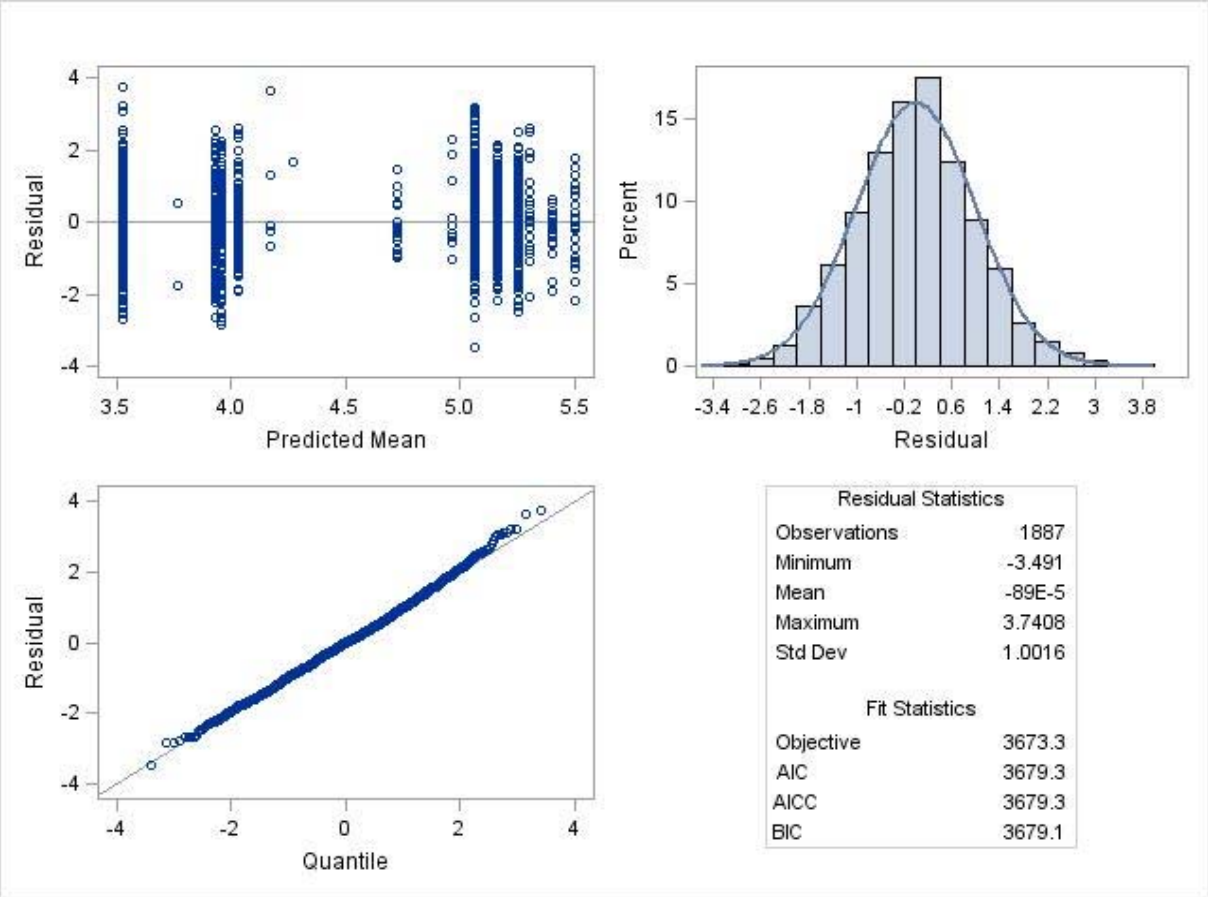

Figure 9: M2 for *Enterobacteriaceae*: Marginal Studentized Residuals Plots

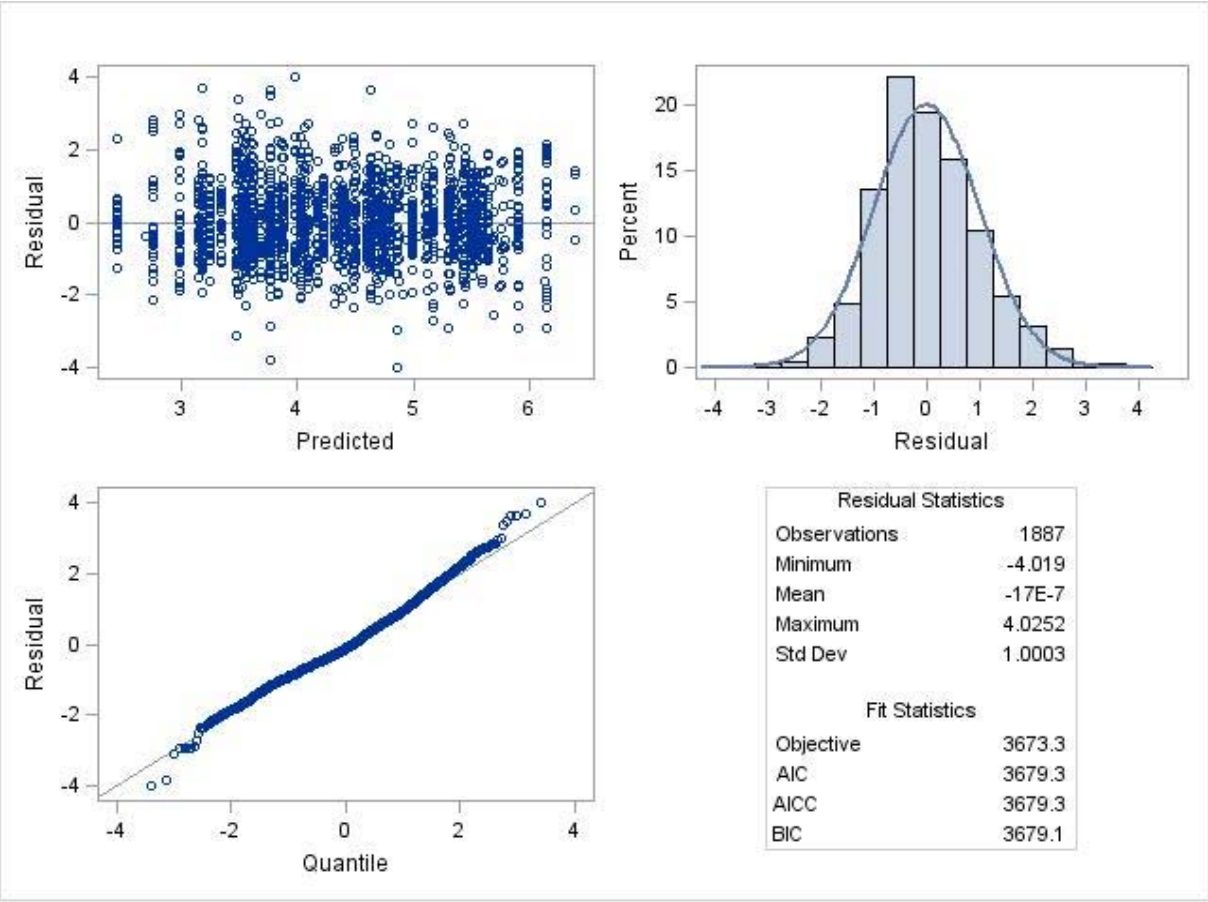

Figure 10: M2 for *Enterobacteriaceae*: Conditional Studentized Residuals Plots

Table 11: M3 for *E. coli*: Significance of fixed effects

		Type 3 Tests of Fixed Effects			
	Variables (short name)	Num DF	Den DF	F Value	Pr > F
	sampInfo	°	°	°	°
	sampMatInfo*sampInfo	°	°	°	°
Batch	batchWeight	2	8.76	11.33	0.0037
	p_rejects _01	1	3.24	11.47	0.0381
	Fit Statistics		Covariance Parameter Estimates		
	-2 Res Log Likelihood	3592.3		Estimate	St. Error
	AIC (smaller is better)	3598.3	$\sigma^2_{\text{int: slaugh}}$	0.01131	0.02629
	AICC (smaller is better)	3598.3	$\sigma^2_{\text{int: batch}}$	0.1734	0.0356
	BIC (smaller is better)	3598.1	σ^2	0.3559	0.01178

° : Variables that cannot be included in the model

Table 12: M3 for *E. coli*: Differences of Least Squares Means

				Average log counts estimate		Difference estimate	Standard Error	p-value	Adjust p-value
batch	batchWeight	2-3 kg	Above 3 kg	3.1182	3.8499	-0.7317	0.1603	0.003	0.0071
		2-3 kg	Below 2 kg	3.1182	3.6279	-0.5097	0.1508	0.0048	0.0071
		Above 3kg	Below 2 kg	3.8499	3.6279	0.222	0.1586	0.1935	0.1935
	p_rejects _01	No	Si	3.1901	3.874	-0.6839	0.2019	0.0381	

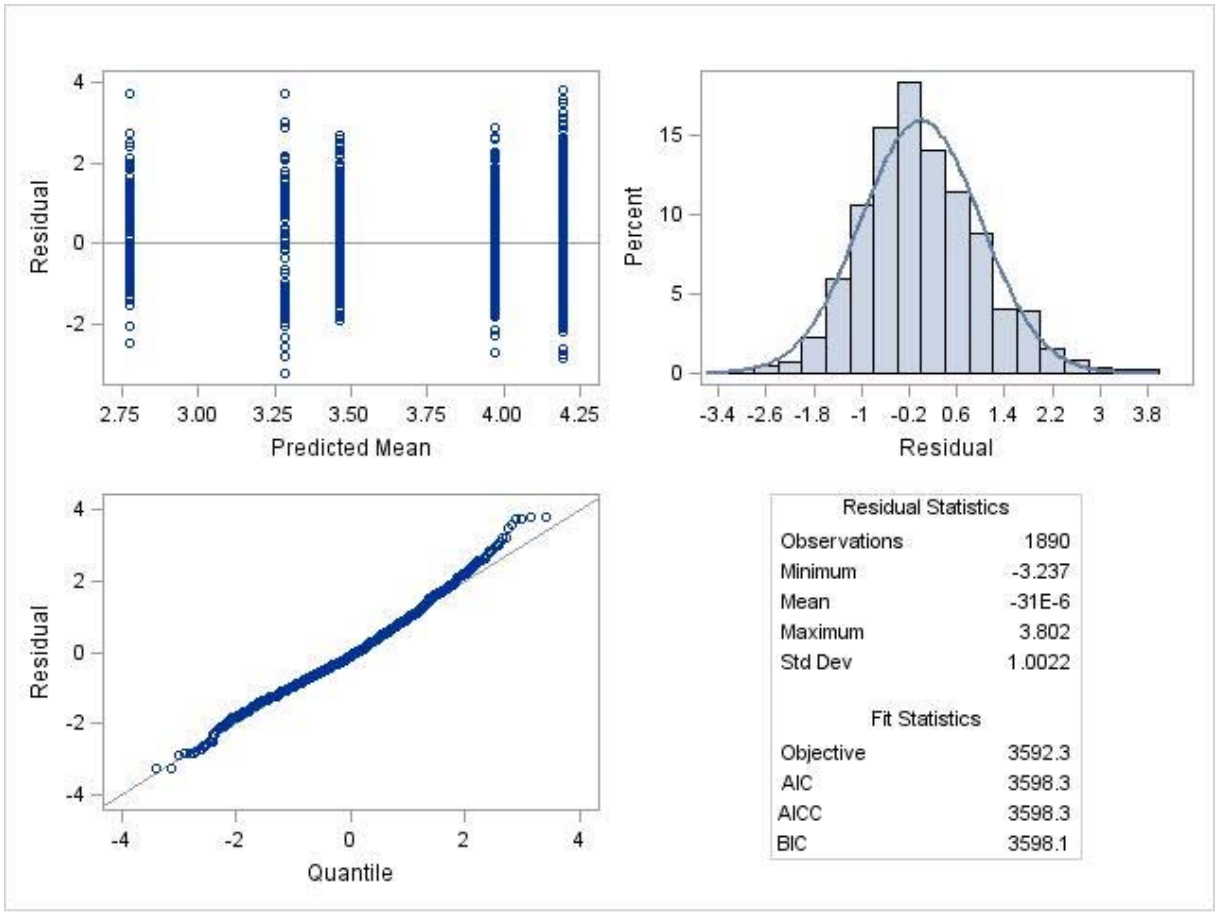

Figure 11: M3 for *E. coli*: Marginal Studentized Residuals Plots

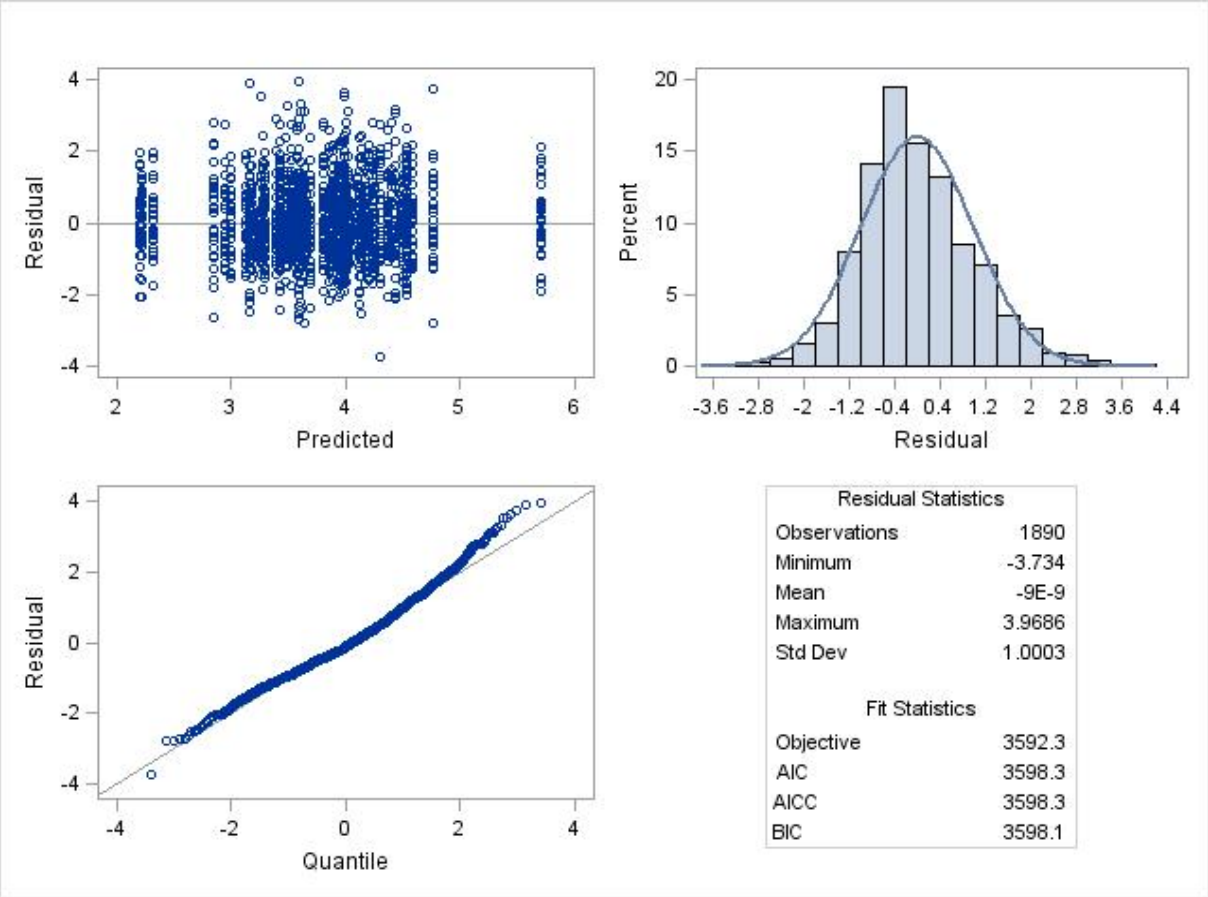

Figure 12: M3 for *E. coli*: Conditional Studentized Residuals Plots

Table 13: M3 for *Enterobacteriaceae*: Significance of fixed effects

		Type 3 Tests of Fixed Effects			
	Variables (short name)	Num DF	Den DF	F Value	Pr > F
	sampInfo	°	°	°	°
	sampMatInfo*sampInfo	°	°	°	°
Batch	batchWeight	2	20.9	7.45	0.0036
	p_rejects _01	1	3.88	8.19	0.0476
	Fit Statistics		Covariance Parameter Estimates		
	-2 Res Log Likelihood	3380.1		Estimate	St. Error
	AIC (smaller is better)	3386.1	$\sigma^2_{\text{int:slaugh}}$	0.04872	0.05051
	AICC (smaller is better)	3386.2	$\sigma^2_{\text{int:batch}}$	0.1595	0.03273
	BIC (smaller is better)	3386	σ^2	0.3172	0.01049

° : Variables that cannot be included in the model

Table 14: M3 for *Enterobacteriaceae*: Differences of Least Squares Means

				Average log counts estimate		Difference estimate	Standard Error	p-value	Adjust p-value
batch	batchWeight	2-3 kg	Above 3kg	3.3225	3.9493	-0.6268	0.1964	0.0062	0.0094
		2-3 kg	Below 2 kg	3.3225	3.908	-0.5855	0.169	0.0015	0.0045
		Above3kg	Below 2 kg	3.9493	3.908	0.04129	0.1854	0.8259	0.8259
	p_rejects _01	No	Si	3.3112	4.1419	-0.8307	0.2903	0.0476	

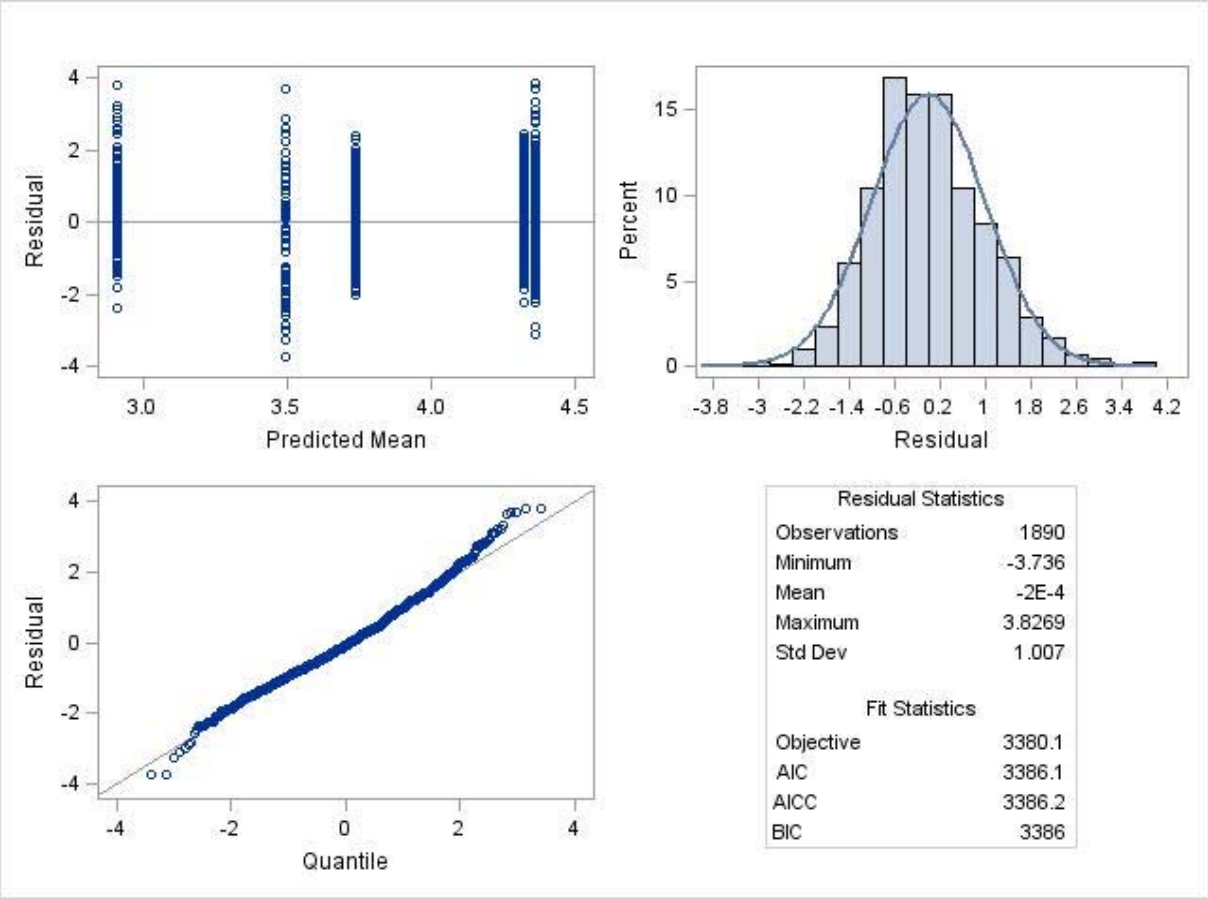

Figure 13: M3 for *Enterobacteriaceae*: Marginal Studentized Residuals Plots

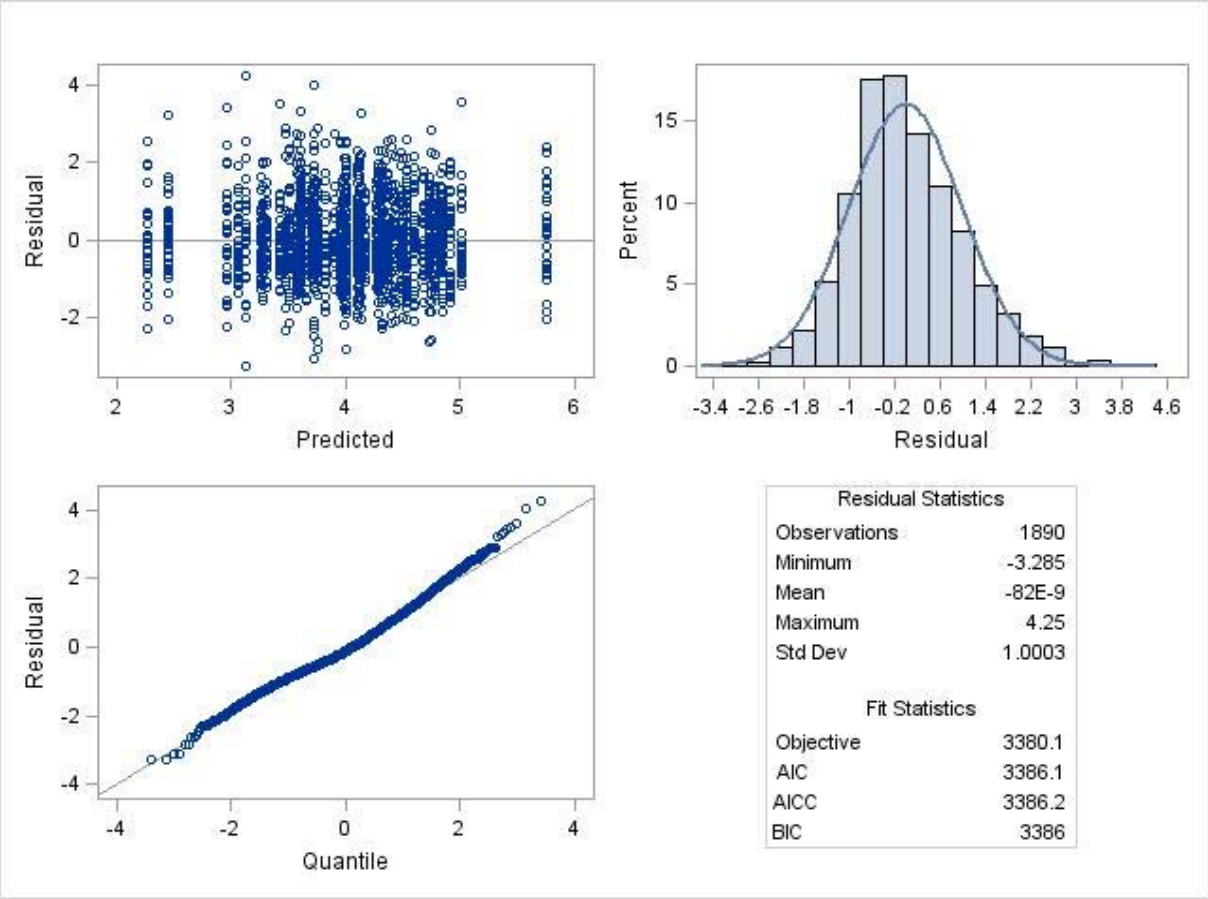

Figure 14: M3 for *Enterobacteriaceae*: Conditional Studentized Residuals Plots

Table 15: M4 for *E. coli*: Significance of fixed effects

		Type 3 Tests of Fixed Effects			
	Variables (short name)	Num DF	Den DF	F Value	Pr > F
	sampInfo	°	°	°	°
	sampMatInfo*sampInfo	°	°	°	°
Batch	batchWeight	2	52.8	2.97	0.0601
	log_counts_POEV	1	51.7	11.96	0.0011
	log_countsPOEV*batchWeight	2	47.7	2.68	0.0791
	Fit Statistics		Covariance Parameter Estimates		
	-2 Res Log Likelihood	3589.1		Estimate	St. Error
	AIC (smaller is better)	3595.1	$\sigma^2_{\text{int:slaugh}}$	0.01131	0.02629
	AICC (smaller is better)	3595.1	$\sigma^2_{\text{int:batch}}$	0.1734	0.0356
	BIC (smaller is better)	3594.9	σ^2	0.3559	0.01178

° : Variables that cannot be included in the model

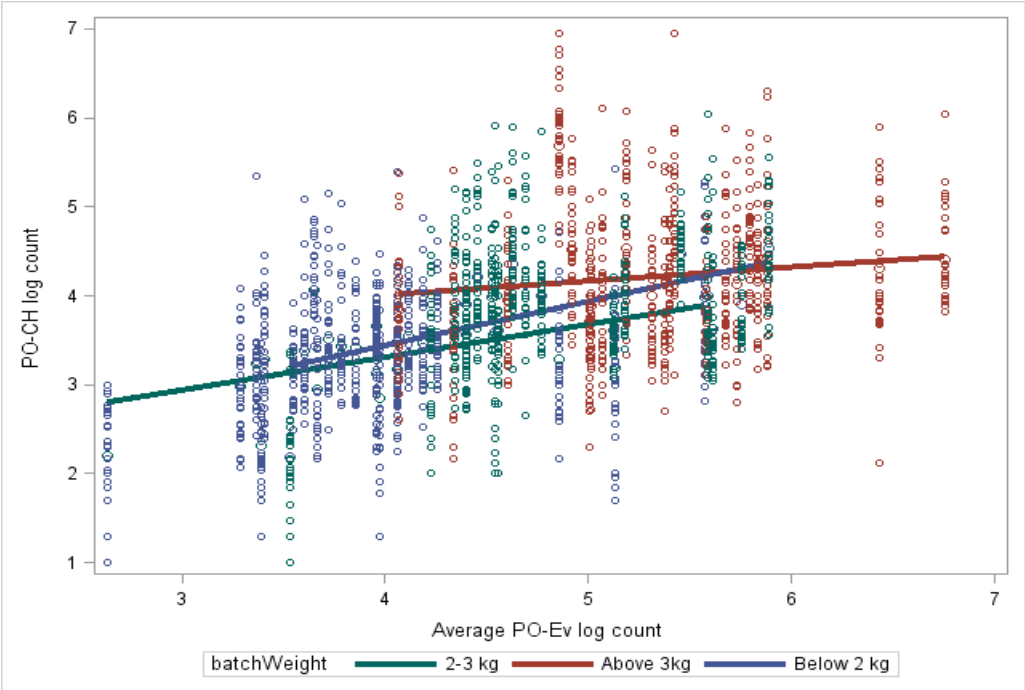

Figure 15: Linear relation between PO-CH log counts of *E. coli* at carcass level and PO-EV log counts average at batch level

Table 16: M4 for *E. coli* Differences of Least Squares Means

				Average log counts estimate		Difference estimate	Standard Error	p-value	Adjust p-value
batch	batchWeight	2-3 kg	Above 3kg	3.5074*	4.0294*	-0.5221	0.2264	0.0257	0.0389
		2-3 kg	Below 2 kg	3.5074*	3.9549*	-0.4476	0.195	0.0259	0.0389
		Above 3kg	Below 2 kg	4.0294*	3.9549*	0.07449	0.2017	0.7139	0.7139

* average count level estimated at the post chilling sampling point for each batch weight category given the average value of counts at the post evisceration sampling point (equal to 4.67 cfu $\log_{10}$/g).

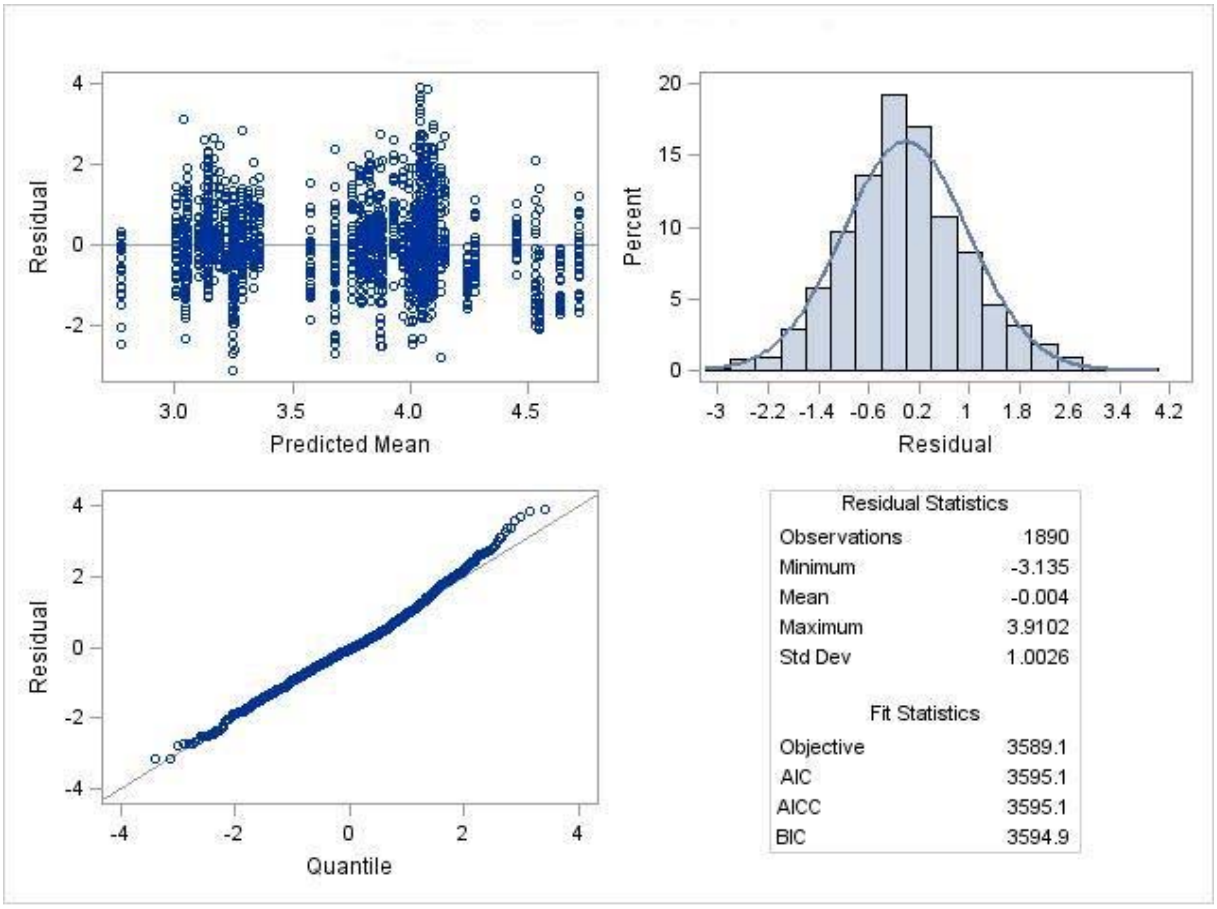

Figure 16: M4 for *E. coli*: Marginal Studentized Residuals Plots

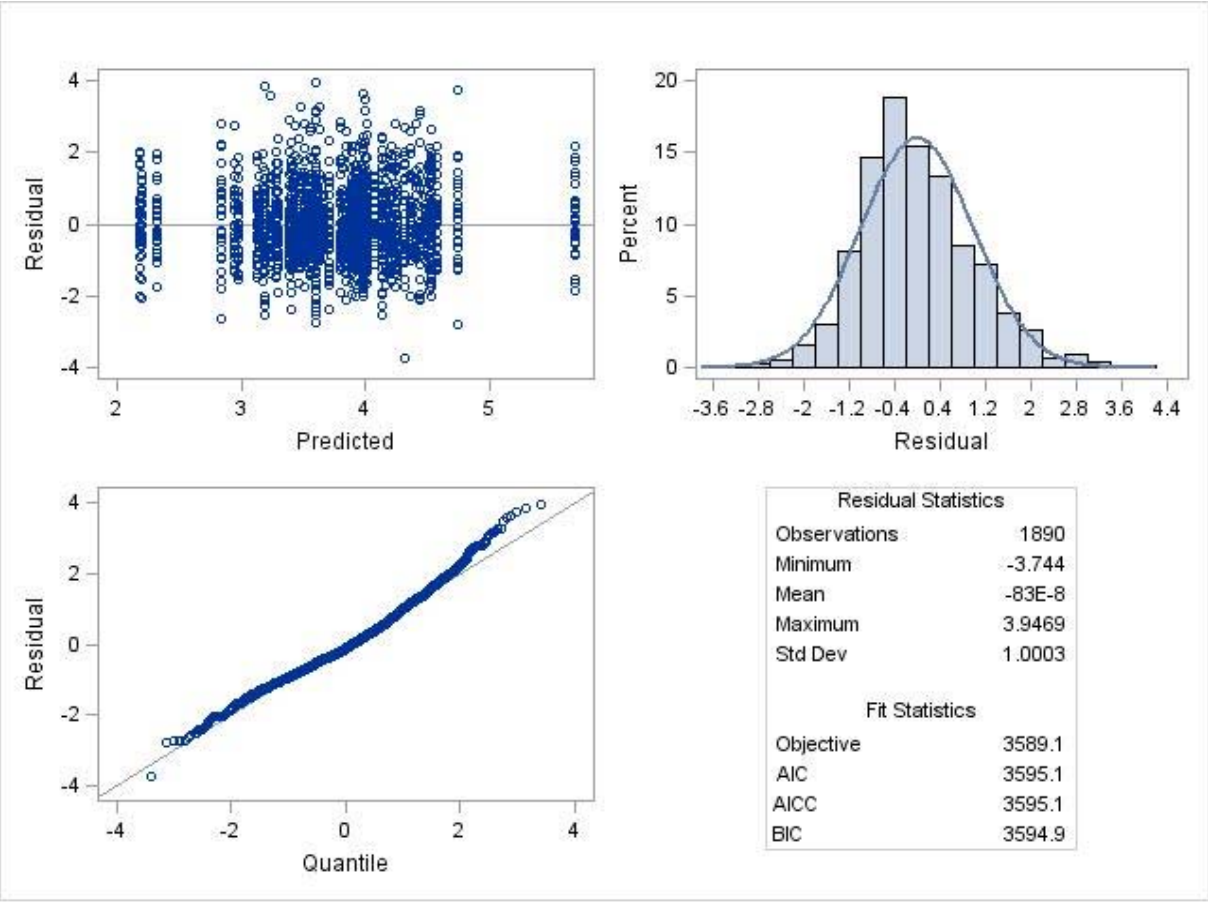

Figure 17: M4 for *E. coli*: Conditional Studentized Residuals Plots

Table 17: M4 for *Enterobacteriaceae* : Significance of fixed effects

		Type 3 Tests of Fixed Effects			
	Variables (short name)	Num DF	Den DF	F Value	Pr > F
	sampInfo	°	°	°	°
	sampMatInfo*sampInfo	°	°	°	°
Batch	batchWeight	2	53.9	3.45	0.039
	log_counts_POEV	1	51.4	18.83	<.0001
	log_countsPOEV*batchWeight	2	49.7	3.29	0.0454

Fit Statistics		Covariance Parameter Estimates		
-2 Res Log Likelihood	3373		Estimate	St. Error
AIC (smaller is better)	3379	$\sigma^2_{\text{int: slaugh}}$	0.0529	0.04478
AICC (smaller is better)	3379	$\sigma^2_{\text{int: batch}}$	0.1305	0.02769
BIC (smaller is better)	3378.8	σ^2	0.3172	0.01049

° : Variables that cannot be included in the model

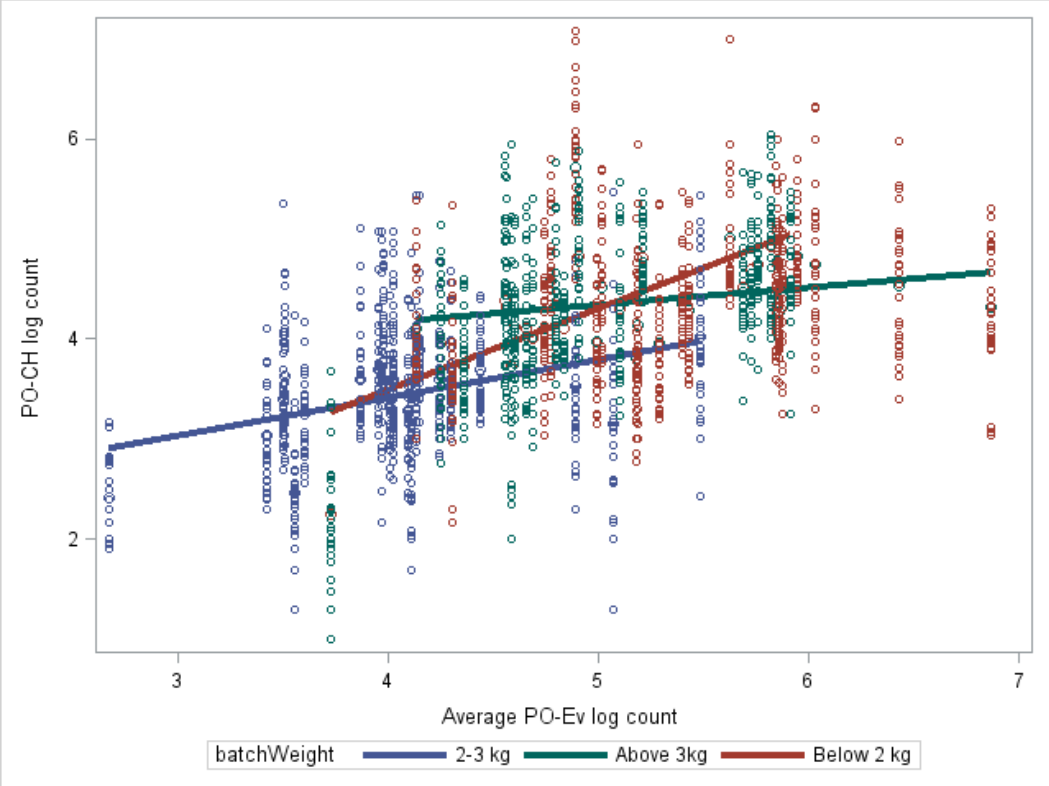

Figure 18: Linear relation between PO-CH log counts of *Enterobacteriaceae* at carcass level and PO-EV log counts average at batch level

Table 18: M4 for *Enterobacteriaceae*: Differences of Least Squares Means

				Average log counts estimate		Difference estimate	Standard Error	p-value	Adjust p-value
batch	batchWeight	2-3 kg	Above 3kg	3.7349*	4.2554*	-0.5204	0.2076	0.016	0.0267
		2-3 kg	Below 2 kg	3.7349*	4.1826*	-0.4476	0.1827	0.0178	0.0267
		Above 3kg	Below 2 kg	4.2554*	4.1826*	0.0728	0.1818	0.6912	0.6912

*: average count level estimated at the post chilling sampling point for each batch weight category given the average value of counts at the post evisceration sampling point (equal to 4.77cfu $\log_{10}$/g).

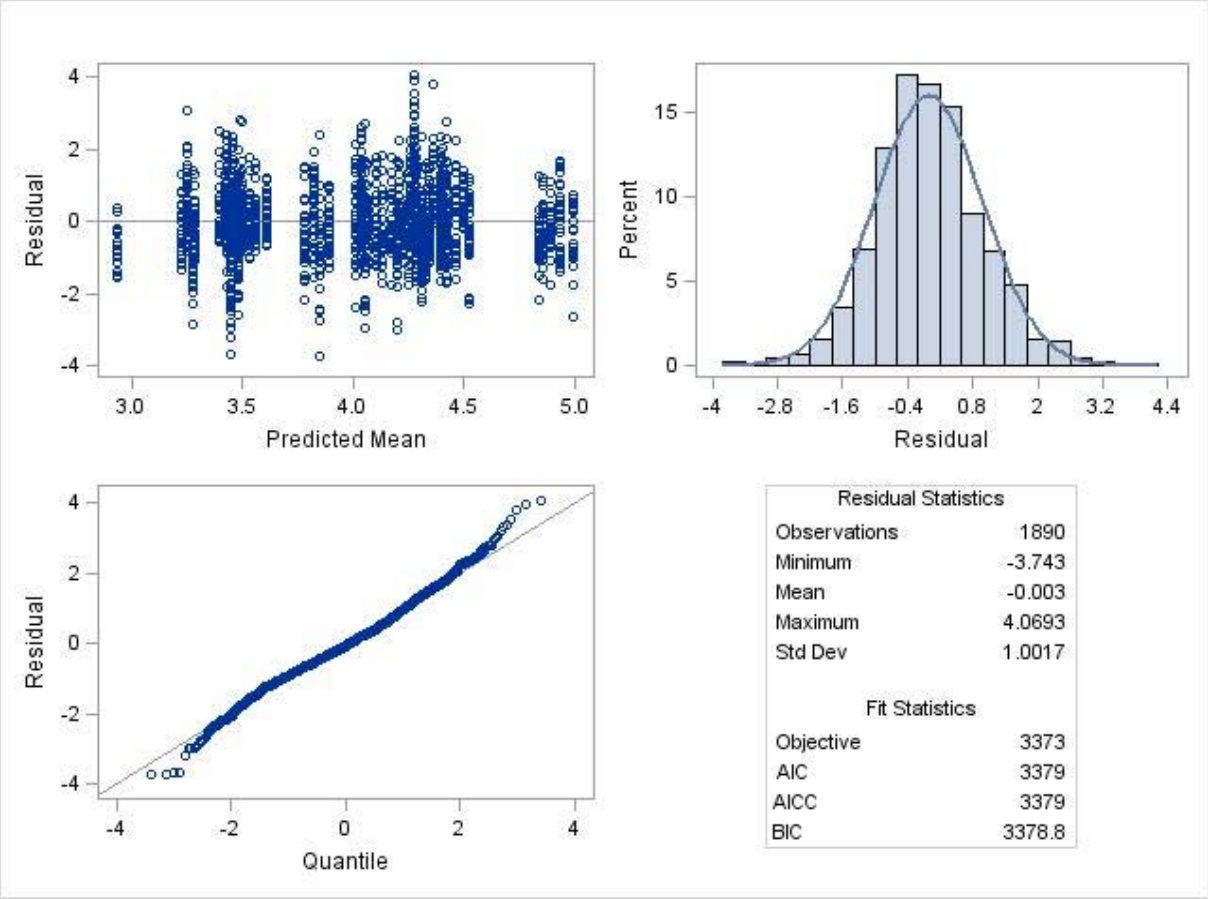

Figure 19: M4 for *Enterobacteriaceae*: Marginal Studentized Residuals Plots

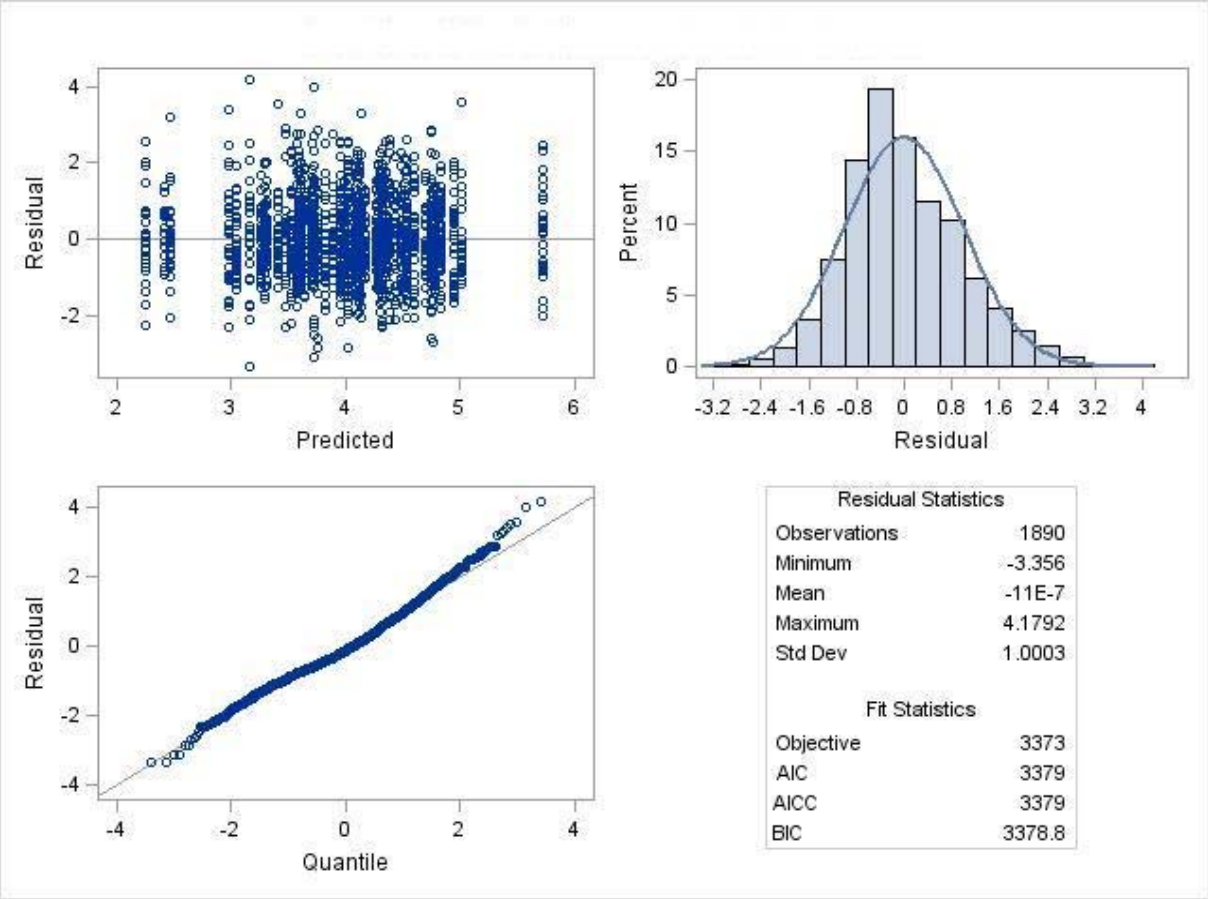

Figure 20: M4 for *Enterobacteriaceae*: Conditional Studentized Residuals Plots